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Original article

Immunohistochemical characterization of the jugular (superior vagal) ganglion in the pig

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Abstract

The study was carried out on three 4-month old female pigs. All the animals were deeply anesthetized and transcardially perfused with 4% buffered paraformaldehyde (pH 7.4). Left and right superior vagal ganglia (SVG) were collected and processed for immunofluorescence labeling method. The preparations were examined under a Zeiss LSM 710 confocal microscope equipped with adequate filter block.

Neurons forming SVG were round or oval in shape with a round nucleus in the center. The majority of them (52%) were medium (M) (31-50 µm in diameter) while 7% and 41% were small (S) (up to 30µm in diameter) or large (L) (above 50 µm in diameter) in size, respectively.

Double-labeling immunofluorescence revealed that SVG neurons stained for CGRP (approx. 57%; among them 37%, 9% and 54% were M, S and L in size, respectively), SP (14.5%; 72.4% M, 3.4% S, 24.2% L), VACHT (26%; 63% M, 24% S and 13% L), GAL (14%; 57% M, 29% S, 14% L), NPY (12%; 53% M, 12% S, 35% L), Met-Enk (5%; 40% M, 6% S and 54% L), PACAP (15%; 52% M, 24% S and 24% L),VIP (6.3%; 67% M, 8% S and 25% L), and NOS-positive (6%; 31% M and 69% L). The most abundant populations of intraganglionic nerve fibers were those which stained for CGRP or GAL, whereas only single SP-, PACAP- or Met-ENK-positive nerve terminals were observed.

Key words: pig, jugular, ganglion, immunohistochemistry

Introduction

The superior ganglion of the vagus nerve, also called the jugular ganglion (JG), was a subject of immunohistochemical studies performed in small laboratory animals, birds and humans. Immunohistochemical properties of neurons forming this ganglion were already described in the rat (Helke and Hill 1988, Silverman and Kruger 1989, Helke and Niederer 1990, Ding et al. 1998, Ichikawa et al. 2007, Hayakawa et al. 2011), mouse (Funakoshi et al. 1989, Dinh et al. 2005a), rabbit and pigeon (Katz and Kar-

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Table 1. Antisera used in the study.

Antigen	Host	Type	Dilution	Cat. No.	Supplier
CGRP	rabbit	polyclonal	1:2000	11535	Cappel
SP	rat	monoclonal	1:150	8450-0505	ABD Serotec, UK
VACHT	rabbit	polyclonal	1:5000	V5387	Sigma
NOS	rabbit	polyclonal	1:2000	11736	Cappel
NPY	rabbit	polyclonal	1:400	NA1233	Biomol
GAL	rabbit	polyclonal	1:2000	RIN 7153	Peninsula Lab.
VIP	mouse	monoclona	1:500	MaVIP	East Acres Biologicals
38-PACAP	rabbit	polyclonal	1:10000	T-4473	Peninsula Lab.
Met-ENK	rabbit	polyclonal	1:500	RPN 1562	Amersham

Secondary antisera

Host	fluorochrom	Dilution	Code	Supplier
Goat-anti-rabbit IgG (H+L)	Alexa Flour 488	1:500	A11008	Invitrogen
Goat-anti-mouse IgG (H+L)	Alexa Flour 488	1:500	A11001	Invitrogen
Goat-anti-rat IgG (H+L)	Alexa Flour 488	1:500	A11006	Invitrogen

ten 1980), chicken (Strobbia et al. 1988), guinea pig (Kummer et al. 1990) and man (Fischer and Hoffmann 1996). These studies have revealed the presence of some different neurotransmitter substances in SVG neurons; among them the most abundant were those immunoreactive to CGRP, SP, VIP, NOS, NPY, enkephalins and Gal. Neurons forming the SVG are involved in the innervation of different cervical and thoracic visceral organs including the heart (Dinh et al. 2005a, Dinh et al. 2005b, Joachim et al. 2006, Hayakawa et al. 2011), gastrointestinal tract (Hayakawa et al. 2012, Hayakawa et al. 2014a) and cranial blood vessels (Uddman et al. 1989, Uddman and Edvinsson 1989).

In the available literature there are some papers dealing with cranial ganglia in the pig. Until now immunohistochemical characteristics of the cilary (Kaleczyc et al. 2005), otic (Lakomy et al. 2002) and pterygopalitine ganglion (Sienkiewicz et al. 1995, Podlasz et al. 2003) were described. The above mentioned ganglia belong to the parasympathetic division of the autonomic nerve system. Immunocytochemical properties of porcine cranial sensory ganglia neurons were also previously investigated (Sienkiewicz et al. 1995, Bulc et al. 2013, Rytel L et al. 2015), but these studies concerned the trigeminal and nodose ganglia.

As clearly seen from the above literature, the data regarding immunohistochemical features of SVG neurons are very limited and have been obtained in birds, laboratory animals and humans, but there is no such information regarding large domestic animals, in this number also the pig. The localization and morphology of the porcine SVG have been described relatively recently (Sienkiewicz and Dudek 2009). However, no information is available concerning neurochemical properties of its nerve elements. Therefore, this study was aimed at investigating the chemical coding of nerve structures in this porcine ganglion.

Materials and Methods

The study was carried out on three 4-month-old female pigs of the Polish Landrace breed, weighing 40-45 kg each. 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). All the pigs were pre-treated with Propionylpromasine (Combelen, Bayer,Germany; 0.4 mg/kg of b.w. i.m.) 30 min before the main anesthetic, Pentobarbital (Vetbutal, Biowet, Poland; 25 mg/kg of b.w.), was given intravenously. Then, they were transcardially perfused with 0.5 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; added extempore), followed by 8-10 l of 4% ice-cold buffered paraformaldehyde (pH 7.4), and the left and right SVG were collected. The tissues were postfixed by immersion in the same fixative for 30 min., rinsed with phosphate buffer (pH 7.4), and transferred to and stored in 18% buffered sucrose solution (pH 7.4) until further processing.

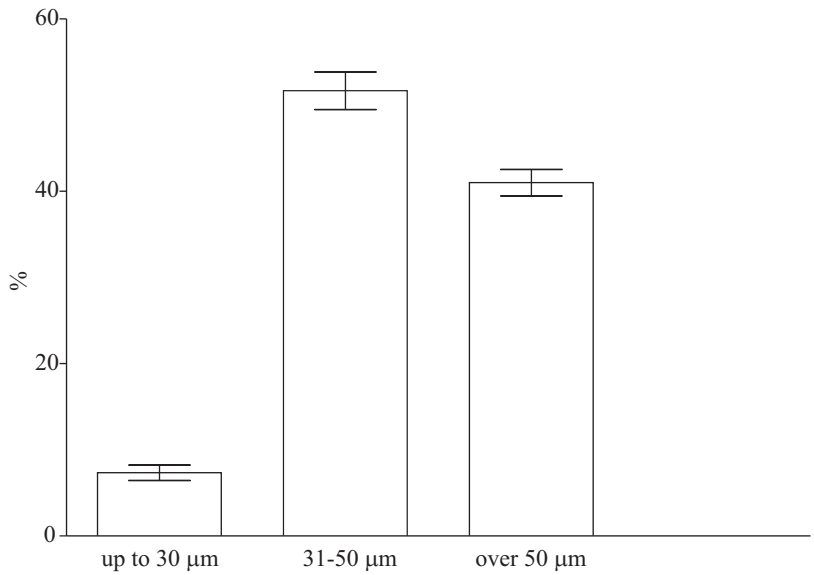

Fig. 1. Mean number of differently sized neurons in the SVG.

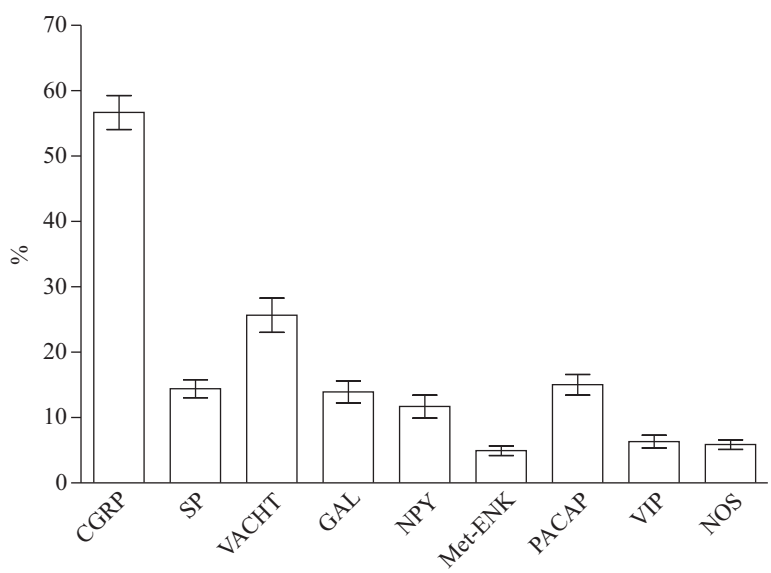

Fig. 2. Percentages of different neuronal subpopulations in the SVG of the pig.

The ganglia were cut into 12-µm-thick cryostat sections, which were processed for the immunofluorescence method on slide-mounted sections. The sections were washed 3 times for 10 min each in PB, incubated 45 min in 10% normal horse serum (NHS, Cappel, Warsaw, Poland) in PBS containing 0.25% Triton X-100 (Sigma, St. Louis, MO, USA), and then incubated overnight at room temperature (RT) with antibodies (Table 1) diluted in PB containing 1% normal swine serum (NSS) and 0.25% Triton X-100. After incubation with the primary antisera, the sections were washed 3 times for:10 min each in PB, and further incubated with the secondary antisera for 1 hour at RT. After incubation, the sections were washed 3 times for 10 min each in PB and cover-slipped with buffered glycerol. Control of specificity of staining was performed by pre-absorption of a diluted antiserum with 20 ng/ml of an appropriate antigen (besides VACHT), which abolished the specific immunoreaction completely. In addition, experiments were carried out in which the primary antiserum was replaced by non-immune serum, or by PBS, to verify the specificity of particular immunoreactions. Preparations were studied, photographed and neurones were measured with a confocal microscope Zeiss LSM 700.

Counting of neurons: to determine percentages of particular neuronal populations, at least 300 of neuronal profiles investigated for each combination of antisera were counted. The sections were collected

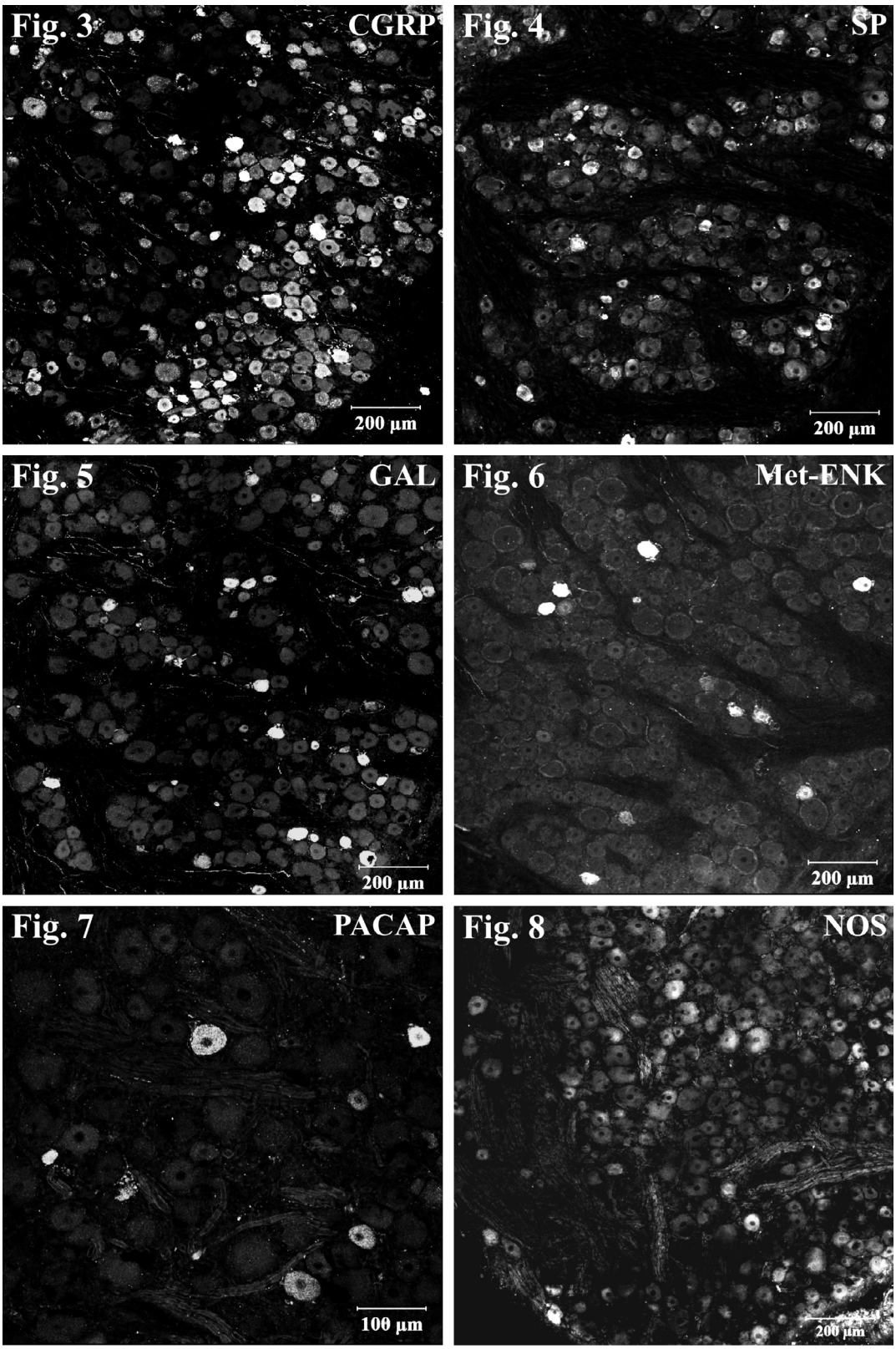

Fig. 3. Numerous CGRP-positive neurons (labelled with Alexa Fluor® 488) in the porcine superior vagal ganglion. Scale bar = 200 µm; Fig. 4. Moderate number of SP-positive neurons (labelled with Alexa Fluor® 488) in the porcine jugular ganglion. Scale bar = 200 µm; Fig. 5. Moderate number of GAL-positive neurons (labelled with Alexa Fluor® 488) in the porcine superior vagal ganglion. Scale bar = 200 µm; Fig. 6. Met-ENK-positive neurons (labelled with Alexa Fluor® 488) in the porcine superior vagal ganglion. Note the presence of immunoreactive nerve fibers. Scale bar = 200 µm; Fig. 7. PACAP-positive neurons (labelled with Alexa Flur® 488) in the porcine superior vagal ganglion. Scale bar = 100 µm; Fig. 8. NOS-positive neurons (labelled with Alexa Fluor® 488) in the porcine superior vagal ganglion. Scale bar = 200 µm.

from different, representative regions of the ganglion (from its upper, middle and lower one-third). To avoid double-counting of the same neurons, appropriate distance (minimum 6 sections = 70 μm) between the sections was maintained. The number of immunolabelled profiles was calculated as a percentage of immunoreactive neurons in regard to all perikarya counted. Statistical analysis was carried out in columns using GraphPad Prism v. 2.0 computer program (GraphPad Software Inc.). All the results are expressed as means ± SEM.

Results

Neurons forming the SVG were round or oval in shape with a round or oval nucleus located in the cell center. Over half of them (51.67±2.19) were medium-sized (31-50 μm), 41% (41.0±1.53) were above 50 μm in diameter (large neurons) and only 7% (7.33±0.88) were small (up to 30 μm) (Fig. 1).

Immunoreactive neurons were distributed evenly throughout the ganglia. Approximately 57% of them were CGRP-immunoreactive (Figs. 2, 3). Moreover, among CGRP-immunoreactive (Fig. 3) neurons 37% were medium, 9% were small and 54% were large in size. The investigation revealed also numerous intraganglionic CGRP-positive nerve fibers. SP (Figs. 2, 4) was expressed by 14.5% of SVG perikarya. SP-positive neurons represented three size categories. Over seventy two percent (72.4%) of them were medium-sized, 3.4% belonged to the population of small perikarya, and 24.2% were large in size. Approximately 26% (25.67%) of neurons were cholinergic in nature (VACHT-IR) (Fig. 2). The population of cholinergic nerve cell bodies consisted of 63%, 24% and 13% medium, small and large in size neurons, respectively. Intraganglionic nerve fibers immunoreactive to VACHT were only single. GAL-immunoreactivity (Figs. 2, 5) was found in 14% of all SVG neurons. Among galaninergic neurons, 57% were medium in size, 29% represented subpopulation of small neurons, whereas only 14% belonged to the class of large nerve cells. Intraganglionic nerve fibers immunoreactive to GAL were moderate in number. NPY-immunoreactivity was present in 12% (11.70%) of all SVG neurons (Fig. 2). It was found that 53% of NPY+ neurons were of medium size, 12% belonged to the subset of small perikarya, and 35% represented large nerve cell bodies. Met-Enk-immunoreactivity (Figs. 2, 6) was expressed by approx. 5% of all the neurons, and single intraganglionic Met-Enk-immnoreactive nerve fibers were found. Among all Met-Enk-positive perikarya 40% were of medium size, 6% were small- and 54% were large-sized neurons. PACAP-immunoreactivity (Figs. 2, 7) was present in 15% of all SVG neurons; among them 52% were medium in size, 24% were small and 24% represented large nerve cells. Only single intraganglionic PACAP-immnoreactive nerve fibers were encountered. VIP-immunoreactivity was observed in 6.3% of all SVG neurons (Fig. 2); among them 67% were medium in size, 8% were of small and 25% were of large size. nNOS-positive nerve cell bodies (6%) represented two size categories: 69% of them belonged to the population of large neurons and the remaining cells were medium in size (Figs. 2, 8).

Disscusion

In some species such as humans, pig, guinea pig and rat, both ganglia of the vagus nerve [proximal (jugular) and distal (nodose)] form clearly separated structures. In the pig, localization of these ganglia has been described in detail (Sienkiewicz and Dudek 2009, Sienkiewicz 2010). The present paper deals with immunohistochemical characteristics of neurons in the porcine SVG. The most abundant subpopulation of the neurons were those immunoreactive to CGRP, which constituted 57% of all ganglionic nerve cells. CGRP-IR neurones were previously disclosed in the rat jugular ganglion (Springall et al. 1987, Helke and Hill 1988, Helke and Niederer 1990, Ding et al. 1998, Hayakawa et al. 2011, Hayakawa et al. 2014b), but unfortunately the above mentioned papers dealt only with neurons supplying particular organs, and it is impossible to refer these results to the entire population of neurons forming the ganglion. Very similar observation was reported in reference to the goat SVG (Kang et al. 2001), where it was found that 57.1% of neurons contained CGRP. Considering porcine cranial sensory ganglia, CGRP-positive neurons were found in the vestibular (Dudek et al. 2012) and nodose (Bulc et al. 2013) ganglia. Eighty two percent of porcine vestibular ganglion neurones displayed immunoreactivity to CGRP, whereas only 12.7% of neurones in the left and 14.9% in the right nodose ganglion were CGRP-IR. These differences can reflect variations between the vagal ganglia in respect to target organs they supply – jugular ganglion neurons innervate organs located in the neck and thoracic cavity, whereas nodose ganglion neurons contribute to the innervation of gastrointestinal tract organs. CGRP, 37-amino acid peptide, is expressed in both peripheral and central neurons. It shows a wide spectrum of biological activity in different tissues. In the motorneurons of the spinal cord, CGRP is a main neurotransmitter responsible for striated muscle contraction; smooth muscles are influenced by CGRP de-

rived from autonomic neurones. It is also a potent vasodilator and important neurotransmitter involved in transmission of pain. It can be assumed that CGRP-IR jugular ganglion neurones are involved in the nociceptive function.

Another neuropeptide expressed in 14.5% of the SVG neurons is SP. This undecapeptide, member of the tachykinin neuropeptide family, discovered in 1931 by Euler and Gaddun (Von Euler and Gaddun 1931), exhibits broad range of biological activities. SP is involved in regulation of vasodilatation, inflammation processes, regulation of mood disorders, anxiety, learning, vomiting, angiogenesis, cell growth, proliferation, and migration, but first of all SP is a key element in pain perception. The presence of SP-positive neurones in the SVG was previously described in goat (Kang et al. 2001), and those neurones constituted a subpopulation amounting up to 48.2%. Similar observations were obtained in rabbit and pigeon (Katz and Karten 1980), guinea pig (Kummer et al. 1990, Kummer et al. 1992) and rat (Ichikawa et al. 1991, Czyzyk-Krzeska et al. 1991b, Finley et al. 1992). Porcine cranial sensory ganglia contain smaller subpopulations of SP-IR neurons; 16.2% and 14.2% of the nerve cell bodies were found in the right and left nodose ganglion (Bulc et al. 2013), and the vestibular ganglion comprises 12% of the perikarya (Dudek et al. 2012). As clearly seen from the above mentioned papers, the sizes of the SP-IR neuronal populations differ significantly, which probably reflects the existence of interspecies differences.

The present study has disclosed that 25.7% of ganglionic neurons in the SVG are immunoreactive to VACHT, and is the first to report the occurrence of cholinergic marker in this ganglion except one contribution (Gauda et al. 2004). This investigation has revealed the presence of VAChT mRNA and immunoreactivity in numerous nerve cells of the nodose-petrosal-jugular ganglion complex, and in a number of microganglion cells embedded in nerve fibers innervating the carotid body, whereas ChAT mRNA was detected in only a few of these cells. Previously the presence of cholinergic neurons was described in dorsal root ganglia (Castrignano et al. 1990, Tata et al. 1994, Yasuhara et al. 2004, Tata et al. 2004, Bellier and Kimura 2007) and also in the porcine vestibular ganglion where more than 50% of the neurons expressed VACHT immunoreactivity (Dudek et al. 2012). The exact role of these neurons needs to be elucidated, but earlier reports entitle us to believe that they are involved in modulation or co-transmission in conducting of stimuli (Baurle et al. 1999) and that they can play an important role in the communication between neurons in sensory ganglia (Tata et al. 2004).

Neuropeptide galanin is a single chain peptide of 29 amino acid residues (30 amino acids in humans). It derives from 123-amino acid protein known as preprogalanin. Galanin was first identified from porcine intestinal extracts in 1983 (Tatemoto et al. 1983). It has a very broad range of physiological functions including nociception, waking and sleep regulation, cognition, feeding, regulation of mood, regulation of blood pressure; it also has roles in neurogenesis and development, and can also act as a trophic factor (Lang et al. 2015). Galanin is linked to a number of diseases including Alzheimer's disease, epilepsy, depression, eating disorders and cancer. It shows also neuroprotective activity following axotomy in the peripheral nervous system and in the brain when seizure activity occurs (Lang et al. 2015). The presence of galanin in the SVG was described in the goat (Kang et al. 2001) and rat (Ichikawa and Helke 1993). In the goat, galaninergic subpopulation of SVG neurons comprises 8.6% of all the nerve cells, whereas in the paper concerning the rat there is no information on the percentage of this neuronal population. In our study we have found a subpopulation of Gal-IR neurons that amounted to approximately 14%. Surprisingly, in the porcine nodose ganglion a small subset of Gal-IR neurons (slightly over 1% in the left and 0.9 % in the right ganglion) was described (Bulc et al. 2013). The presence of galanin was also confirmed in the nodose porcine ganglion by Zalecki (Zalecki 2014). However, in another porcine sensory cranial ganglion, vestibular ganglion, a comparable population (15%) of Gal-IR perikarya was previously disclosed (Dudek et al. 2012).

NPY is a 36-amino acid neuropeptide that acts as a neurotransmitter in the central and peripheral nervous system. Primarily it serves as a strong regulator of vascular tone. In the brain, NPY influences several functions: increases food intake and storage of energy as fat, reduces anxiety and stress, affects the circadian rhythm, reduces voluntary alcohol intake, lowers blood pressure, and controls epileptic seizures (Pedrazzini et al. 2003, Decressac and Barker 2012). In the sensory nervous system, NPY is involved in the reduction of pain perception (Yalamuri et al. 2013). Immunoreactivity to NPY has been found in dorsal root and trigeminal ganglia neurons (DeLeon et al. 1994). In the rat, scarce neurons located in the nodose and petrosal ganglia express preproNPY mRNA (Czyzyk-Krzeska et al. 1991b) Transection of the vagus nerve results in the increased number of NPY-IR and NPY mRNA-containing neurons in the nodose and petrosal ganglia in the rat (Zhuo et al. 1997). As far as the porcine cranial sensory ganglia are concerned, there is only one report confirming the presence of a population of NPY-IR nerve cell bodies

amounting up to 14.7% of all the ganglionic neurons (Dudek et al. 2012), which is consistent with our present results.

Met-enkephalin is a pentapeptide which was discovered and characterized by John Hughes and Hans Kosterlitz (Hughes et al. 1975) during studies on endogenous ligands of opioid receptors. This peptide is preliminary involved in the regulation of nociception (Przewłocki and Przewłocka 2001) by inhibition of substance P release (Chang et al. 1989). In situ hybridization study showed the expression of mRNA for preproenkephalin A in primary sensory neurons of the rat petrosal ganglion (Czyzyk-Krzeska et al. 1991a). The presence of met-ekephalin-IR neurons was reported in dorsal root and trigeminal ganglia neurones in the rat (Quartu et al. 1990) as well as in the porcine vestibular ganglion (Dudek et al. 2012) which contained a fivefold larger population (25%) of the neurones than that observed in the present study. This difference probably reflects target specific variations in the chemical coding between neurons supplying different organs.

PACAP, a peptide isolated from ovine hypothalami, is a member of the VIP- glucagon/secretin superfamily, and occurs in two forms: PACAP-38 and PACAP-27 (Arimura 1992). This substance plays an important role in nociception (Tajti et al. 2015). The presence of PACAP in the jugular ganglion was described in the rat (Mulder et al. 1995). Studies performed on the nodose ganglion in the pig have revealed that 67.25% of the neurones in the right, and 66.5% in the left ganglion express immunoreactivity to this peptide (Rytel L et al. 2015), whereas the present study has established that in the porcine PGNV PACAP-IR neurones represent only 15% of all ganglionic nerve cells.

Another polypeptide that belongs to a glucagon/secretin superfamily is VIP. This 28 amino acid peptide was first isolated from the porcine gut in 1970 (Said and Mutt 1970). Among a broad spectrum of biological activities, especially as concerns the digestive system, it also plays an important role in nociception (Yeomans et al. 2003, Schytz et al. 2010). In the nodose and petrosal ganglia, only a few VIP-IR (Lundberg et al. 1978, Helke and Hill 1988, Helke and Rabchevsky 1991, Zhuo et al. 1997) or VIP mRNA-containing (Zhuo et al. 1995) neurons can be detected. In the porcine sensory nodose ganglion, 40% of neurones exhibited immunoreactivity to VIP (Rytel L et al. 2015), but only less than 20% VIP-positive nerve cells were observed in the vestibular ganglion (Dudek et al. 2012).

Nitric oxide synthase is an enzyme producing gas molecule NO from arginine. This widely distributed enzyme occurs in animal organisms in three forms and plays a very important role in different functions. The endothelial isoform (eNOS) of the enzyme is a primary signal generator in the control of vascular tone. The inducible isoform (iNOS) produces large amounts of NO as a defense mechanism in the response of the body to attack by parasites, bacterial infection, and tumor growth. Neuronal (nNOS) is an important factor in modulating physiological functions such as learning, memory, and neurogenesis. It mediates long-term regulation of synaptic transmission (long-term potentiation, long-term inhibition). In the peripheral nervous system nNOS is responsible for a decrease in the tone of various types of smooth muscle including those found in blood vessels (Forstermann and Sessa 2012). This enzyme is also involved in the regulation of nociception (Nazeri et al. 2014). The presence of nNOS in sensory ganglia of the vagus nerve was confirmed in the rat with histochemistry and immunohistochemistry (Yamamoto et al. 2003). Porcine cranial sensory ganglia have been found to contain NADPH-diaphorase activity (Sienkiewicz et al. 1995) and immunoreactivity to nNOS (Dudek et al. 2012, Bulc et al. 2013). The vestibular ganglion contain 12% subpopulation of nNOS-IR nerve cells (Dudek et al. 2012), whereas 18.7 and 13.3% of the immunoreactive neurons have been determined in the left and right nodose ganglion, respectively. The present study has demonstrated the occurrence of a slightly smaller subpopulation of nNOS-conatining neurons (6%) in the porcine jugular ganglion.

The present paper describes for the first time immunohistochemical properties of neurons in the porcine jugular ganglion. The study has revealed the occurrence of neurons immunoracative to CGRP, SP, VACHT, GAL, NPY, Met-Enk, PACAP,VIP and NOS. Considering the previously obtained functional data it can be assumed that all these substances are involved in transduction of sensory stimuli.

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