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Original article

Immunohistochemical characterization of nerve elements in porcine intrinsic laryngeal ganglia

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Abstract

The present study investigated the chemical coding of neurons and nerve fibres in local laryngeal ganglia in pigs (n=5) using double-labelling immunohistochemistry. Virtually all the neurons were cholinergic in nature (ChAT- or VAChT-positive). Only very solitary, small nerve cells (presumably representing interneurons) stained intensely for adrenergic marker, DβH. Many neurons also contained immunoreactivity for NOS (91%), VIP (62.7%), NPY (24.7%), galanin (10%), SP (1.3%) and CGRP (5.3%). No neurons expressing somatostatin or Leu-enkephalin were observed. Nearly all the neuronal somata were densely supplied with varicose cholinergic nerve terminals, which presumably represented preganglionic axons, and some of them were also closely apposed with CGRP- and/or SP-positive varicose nerve endings, which were putative collaterals of extrinsic primary sensory fibres. In conclusion, this study has revealed that intrinsic neurons in the porcine larynx, like in many other mammalian species studied, should be classified as parasympathetic cholinergic neurons expressing biologically active substances, predominantly NOS and VIP. Furthermore, they are likely to receive inputs from not only preganglionic neurons but also primary sensory nerve cells. Finally, it appears that the information on the occurrence of the local laryngeal ganglia should be regularly included in textbooks dealing with the cranial portion of the parasympathetic nervous system in mammals.

Key words: larynx, intrinsic neurons, pig, immunohistochemistry, biologically active substances

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Introduction

The innervation of the mammalian larynx is complex and involves afferent and efferent (including autonomic) components. These pathways are mostly accomplished through two branches of the vagus nerve, the superior and inferior (in humans; in animals the cranial and caudal, respectively) laryngeal nerve which is the terminal part of the recurrent laryngeal nerve. The autonomic component includes intramural ganglia regularly associated with the final branches of the laryngeal nerves. The occurrence of cellular clusters resembling neurons accompanying the branches of laryngeal nerves in humans and some other mammalian species has been reported by many investigators, and the first contributions dealing with this problem came into view already in the early 1900s (Grynfelt and Hedón 1907, 1908, Elze 1923, Lemere 1932, Rubatelli 1934, Kleinsasser 1964, Afifi 1971, Ramaswamy et al. 1994, Maranillo et al. 2008). However, the exact nature of these cells and thus, their aggregates was not fully recognized for a long period. Some authors suggested that they represent paraganglia consisting of cells having chemoceptive function (Watzka 1963, Kleinsasser 1964). On the other hand, investigations performed in animals, rats (Luts et al. 1990, Krekel et al.1990, Shimazaki et al. 1995, Hisa et al. 1996b, Robertson et al. 1998, Nakanishi et al. 1999, Koike et al. 2004, Okano et al. 2006), cats (Tsuda et al. 1992, Tanaka et al. 1993a, Yoshida et al. 1993, Shimazaki et al. 1995), dogs (Shimazaki et al. 1995, Hisa et al. 1996a, Yamamoto et al. 1998, Koike and Hisa 1999), guinea-pigs (Shimazaki et al. 1995), rabbits (Shimazaki et al. 1995) and horses (Corcoran et al. 1999) with the use of histo- and immunohistochemical methods have provided strong evidence that these cells are neurons containing some biologically active substances. Furthermore, considering the chemical coding of local laryngeal neurons it is generally assumed that they are parasympathetic nerve cells as they express cholinergic markers (choline acetyltransferase, ChAT, or acetylcholinesterase, AChE) and some other substances typical of cholinergic-parasympathetic nerve structures, such as vasoactive intestinal polypeptide (VIP) or nitrergic markers (nitric oxide synthase, NOS or NADPH-diaphorase, NADPHd).

The present study investigated the chemical coding of neurons and nerve fibres in porcine local laryngeal ganglia since (1) the pig has become a critically important experimental animal in biomedical research (Swindle et al. 2012), and (2) there is still lack of morphological and immunohistochemical data concerning the porcine ganglionic structures.

Materials and Methods

The tissues under study were collected from 5 adult, 6 months old, male pigs. Prior to tissue collection, all the animals were anaesthetized and then sacrificed by exsanguination (via the cranial vena cava). After removal of the entire larynx, branches of the left and right cranial (CLN) and recurrent (RLN) laryngeal nerves were carefully prepared along their course to the mucosal or muscular endings, dissected out and immersed in 4% buffered paraformaldehyde (pH. 7.4) for 1 h.

Then, the tissues were rinsed several times with phosphate buffer (pH 7.4) and transferred to and stored in 18% buffered (pH 7.4) sucrose solution until further processing. The tissues were cut into 10 μm-thick cryostat serial sections and processed for double-labeling immunofluorescence (according to an earlier described method; Sienkiewicz et al. 2010) to localize intramural nerve structures and to study their chemical coding using antibodies (listed in Table 1) against ChAT, vesicular acetylcholine transporter (VAChT), dopamine β hydroxylase (DβH), NOS, VIP, neuropeptide Y (NPY), galanin (GAL), somatostatin (SOM), substance (SP) and calcitonin gene-related peptide (CGRP). Because virtually all the neurons stained for the cholinergic markers [only very solitary small perikarya, most likely representing interneurons - small intensely fluorescent (SIF) cells, stained for DβH], in most cases each mixture of primary antibodies applied contained antibodies against ChAT or VAChT (to easily recognize the nerve structures and to distinguish them from non-neural elements) and those against one of the biologically active substances mentioned.

Some sections adjacent to those selected for immunohistochemical investigations were stained with hematoxylin and eosin.

The sections labelled were mounted with carbonate-buffered glycerol (pH 8.6) and viewed under a Zeiss Axiophot microscope equipped with epi-fluorescence and an appropriate filter set. The colocalization patterns of the substances within the nerve elements were analysed either directly by interchanging filters or indirectly by means of comparison of the consecutive sections. They were also investigated and images were recorded with a Zeiss LSM 700 confocal laser scanning microscope (Zeiss, Jena, Germany), using a 20 × 0.8 plan-apochromat objective lens and ZEN Software 2009. Channels were scanned consecutively to avoid cross-talk.

Standard controls, i.e. preabsorption 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), and omission and replacement of all primary antisera

Table 1. Antisera used in the study.

Primary antisera					
Antigen	Host	Type	Dilution	Cat. no.	Supplier
ChAT	Goat	Polyclonal	1:50	AB144P	Millipore, Temecula, CA, USA
VACHT	Rabbit	Polyclonal	1:5000	V5387	Sigma-Aldrich, Saint Louis, MI, USA
CGRP	Rabbit	Polyclonal	1:2000	11535	Cappel, Aurora, OH, USA
NOS	Rabbit	Polyclonal	1:2000	11736	Cappel, Aurora, OH, USA
NOS	Mouse	Monoclonal	1:100	N2280	Sigma-Aldrich, Saint Louis, MI, USA
SOM	Rabbit	Polyclonal	1:200	LSC39186	Lifespan, Seattle, WA, USA
SP	Rat	Monoclonal	1:200	8450-0505	Biorad
Leu-5-Enk	Mouse	Monoclonal	1:500	4140-0355	AbD Serotec, Oxford, UK
NPY	Rabbit	Polyclonal	1:400	NA1233	Biomol, Exeter, UK
DßH	Rabbit	Polyclonal	1:500	DZ1020-0050	Biomol, Exeter, UK
DßH	Mouse	Monoclonal	1:500	MAB308	Millipore, Temecula, CA, USA
VIP	Rabbit	Polyclonal	1:2000	ab22736	Abcam, Cambridge, UK
VIP	Mouse	Monoclona	1:500	MaVIP	East Acres Biologicals, Southbridge, MA, USA
GAL	Rabbt	Polyclonal	1:2000	AB2233	Sigma

Secondary antisera				
Host	Fluorochrom	Dilution	Code	Supplier
Donkey anti goat IgG (H+L)	Alexa Flour 546	1:1000	A11056	Invitrogen, Paisley, UK
Donkey-anti-rabbit IgG (H+L)	Alexa Fluor 488	1:1000	A21206	Invitrogen, Paisley, UK
Donkey-anti-mouse IgG (H+L)	Alexa Fluor 488	1:1000	A21202	Invitrogen, Paisley, UK
Goat-anti-mouse IgG (H+L)	Alexa Flour 488	1:1000	A11001	Invitrogen, Paisley, UK
Goat-anti-rabbit IgG (H+L)	Alexa Flour 568	1:1000	A11011	Invitrogen, Paisley, UK
Goat-anti-rabbit IgG (H+L)	Alexa Flour 555	1:1000	A21428	Invitrogen, Paisley, UK
Goat anti rat IgG (H+L)	Aleksa Fluor 488	1:1000	A11006	Invitrogen, Paisley, UK

by non-immune sera were applied to test antibody and method specificity.

To determine percentages of the neuronal populations, at least 100 ChAT- or VAChT-positive neuronal profiles investigated for the expression of one of the other biologically active substances were counted in tissues collected from every animal. To avoid double-counting of the same neurons, appropriate distance (minimum 5 sections = 50 µm) between the sections was maintained. The number of immunolabelled profiles was calculated as a percentage of the immunoreactive neurons in relation to all cholinergic perikarya counted.

Results

Immunostainings revealed the presence of many neurons associated with branches of CLN and RLN. Most of them were aggregated forming smaller (containing few perikarya) or larger (containing several to dozens of perikarya) clusters of nerve cells (Figs. 1-6). Solitary neurons were also encountered within nerve bundles traversing the laryngeal tissues. The distribution of intralaryngeal ganglia resembled that described by Maranillo et al. (2008) in humans. The largest ganglion, presumably related to that mentioned by Maranillo et al. (2008), associated with the branch of the superior laryngeal nerve innervating the vestibule, comprised more than one hundred ChAT- or VAChT-positive neuronal profiles in one section (Figs. 1, 2).

Double-labelling immunohistochemistry revealed that virtually all the neurons were cholinergic in nature (ChAT- or VAChT-positive; Figs. 1-4, 6). Only very solitary, small nerve cells stained intensely for DβH. ChAT-immunoreactivity appeared as homogeneous staining in the cytoplasm of a nerve cell body (Figs. 3, 4). This type of staining was similar to that of NOS or SP (Fig. 1, 5). The pattern of VAChT--immunolabelling differed from that of ChAT. The VAChT-IR material often represented "clumps" distributed within the cytoplasm (Figs. 1, 2, 6), frequently closer to and around the nucleus of a nerve cell body. This pattern of the immunolabelling resembled that of VIP, NPY, GAL, or CGRP (Figs. 2, 3, 4, 5).

Many neurons contained also immunoreactivity for NOS (91%), VIP (62.7), NPY (24.7%), GAL (10%),

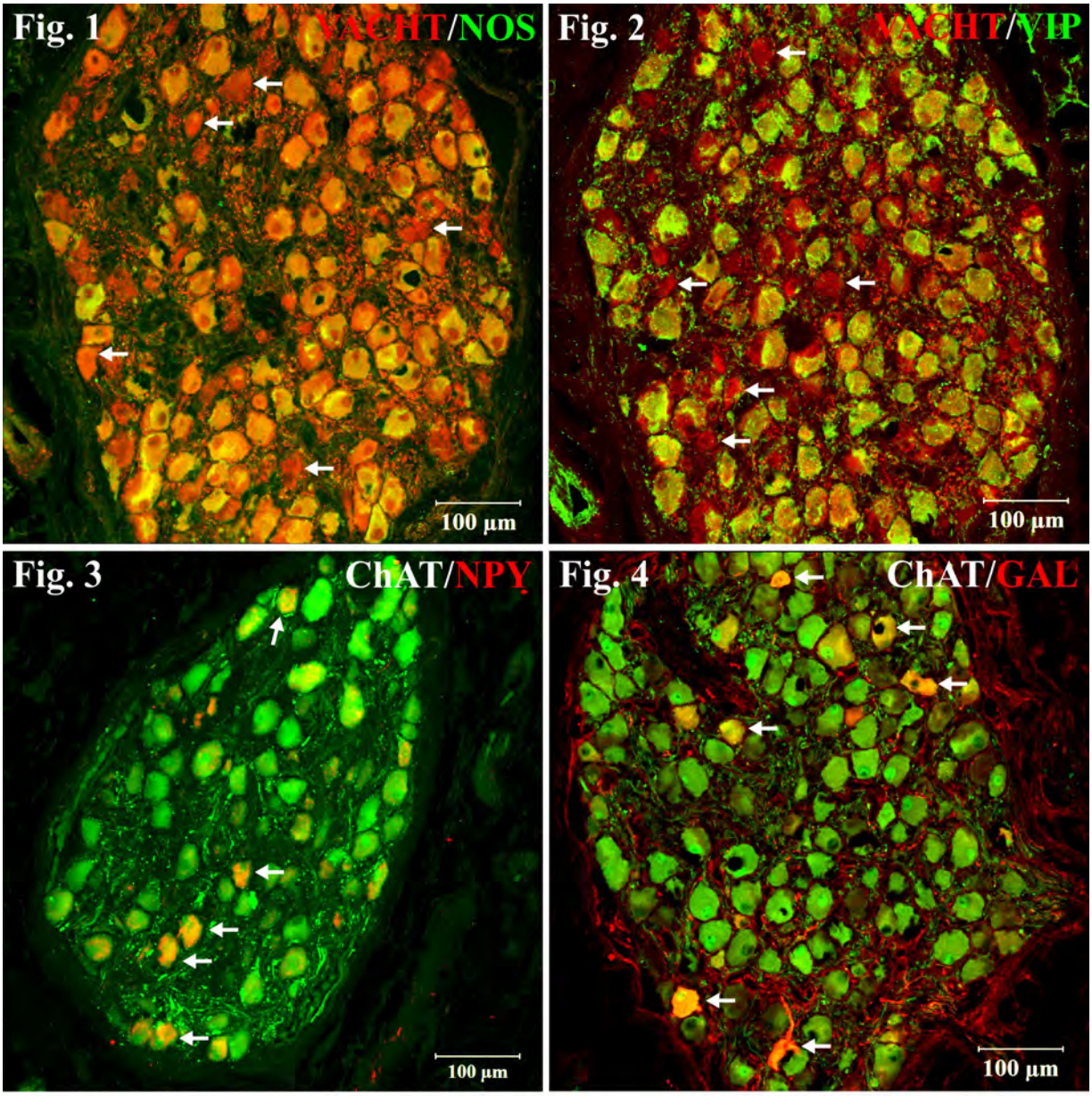

Fig. 1. Confocal laser scanning microscope image showing the distribution of nitric oxide synthase-positive (NOS)+ [green; Alexa 488 visualization (A488)] and vesicular acetylcholine transporter (VAChT)+ [red; Alexa 555 visualization (A555)] neurons in a section from a porcine intramural laryngeal (PIL) ganglion; green and red channels were digitally superimposed (GRDS). Nearly all the neurons stained for NOS and were surrounded by cholinergic varicose nerve fibres (arrows show solitary NOS-negative neuronal somata).

Fig. 2. Confocal laser scanning microscope images showing the distribution of vasoactive intestinal polypeptide (VIP)+ (green; A488) and VAChT+ (red; A555) neurons in a section from PIL ganglion; GRDS. Most of the neurons stained for VIP (arrows show solitary VIP-negative neuronal somata).

Fig. 3. Confocal laser scanning microscope images showing the distribution of choline acetyltransferase (ChAT)+ (green; A488) and neuropeptide Y (NPY)+ (red; A555) neurons in a section from PIL ganglion; GRDS. A moderate number of the neurons stained for NPY (arrows).

Fig. 4. Confocal laser scanning microscope images showing the distribution of ChAT+ (green; A488) and galanin (GAL)+ (red; A555) neurons in a section from PIL ganglion; GRDS. A small number of the neurons stained for GAL (arrows).

SP (1.3%) and CGRP (5.3%) (Fig. 7). No neurons expressing SOM or Leu-Enk were found.

Nearly all the nerve cell bodies were closely apposed by numerous varicose VAChT-positive nerve fibres often forming “baskets” surrounding particular cell somata (Figs. 1, 2). Other prominent populations of the nerve terminals were those stained for SP and/or CGRP. These axons supplied many perikarya also frequently forming “baskets” around the nerve cell bodies (Fig. 5).

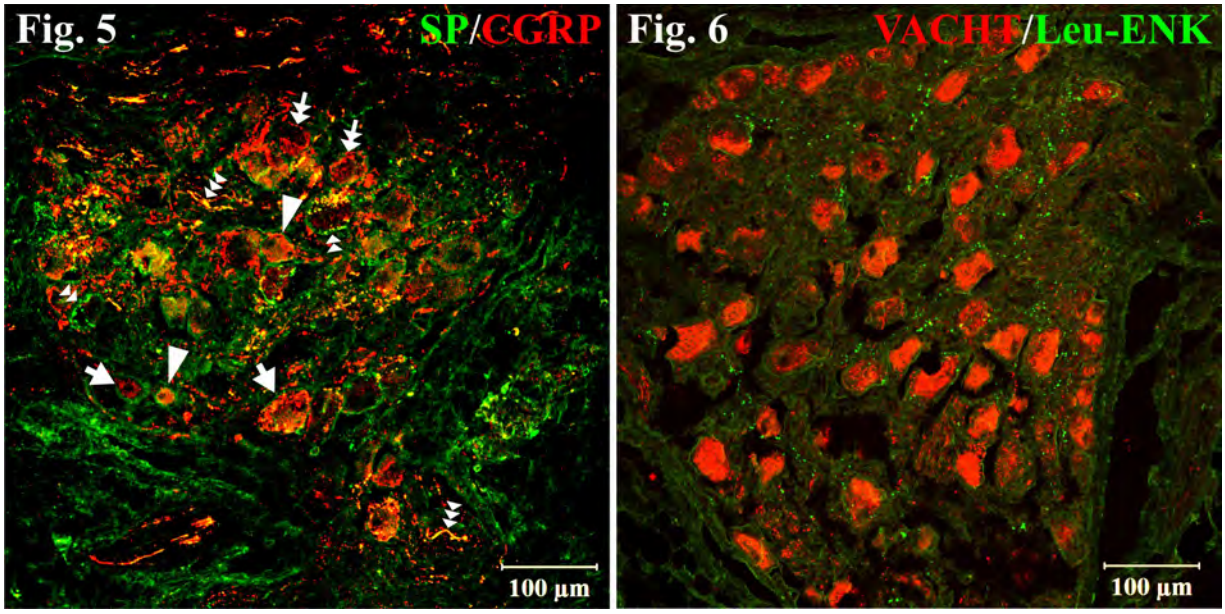

Fig. 5. Confocal laser scanning microscope images showing the distribution of substance P (SP)+ (green; A488) and calcitonin gene-related peptide (CGRP)+ (red; A555) nerve fibres in a section from PIL ganglion; GRDS. Many SP- or CGRP-negative, some SP+ or CGRP+ (thick arrows) and SP+/CGRP+ (arrowheads) neurons were apposed by CGRP+ (double arrows), SP+ (double arrowheads) or CGRP+/SP+ (triple arrowheads) varicose nerve fibres, which often formed "baskets" consisting of axons belonging to even all three populations of the nerve terminals.

Fig. 6. Confocal laser scanning microscope images showing the distribution of Leu-enkephalin (Leu-Enk)+ (green; A488) and VAChT+ (red; A555) nerve structures in a section from PIL ganglion; GRDS. All the neurons stained for VAChT but were Leu-Enk-negative, however some neuronal somata were closely apposed by single Leu-Enk+ varicose nerve endings.

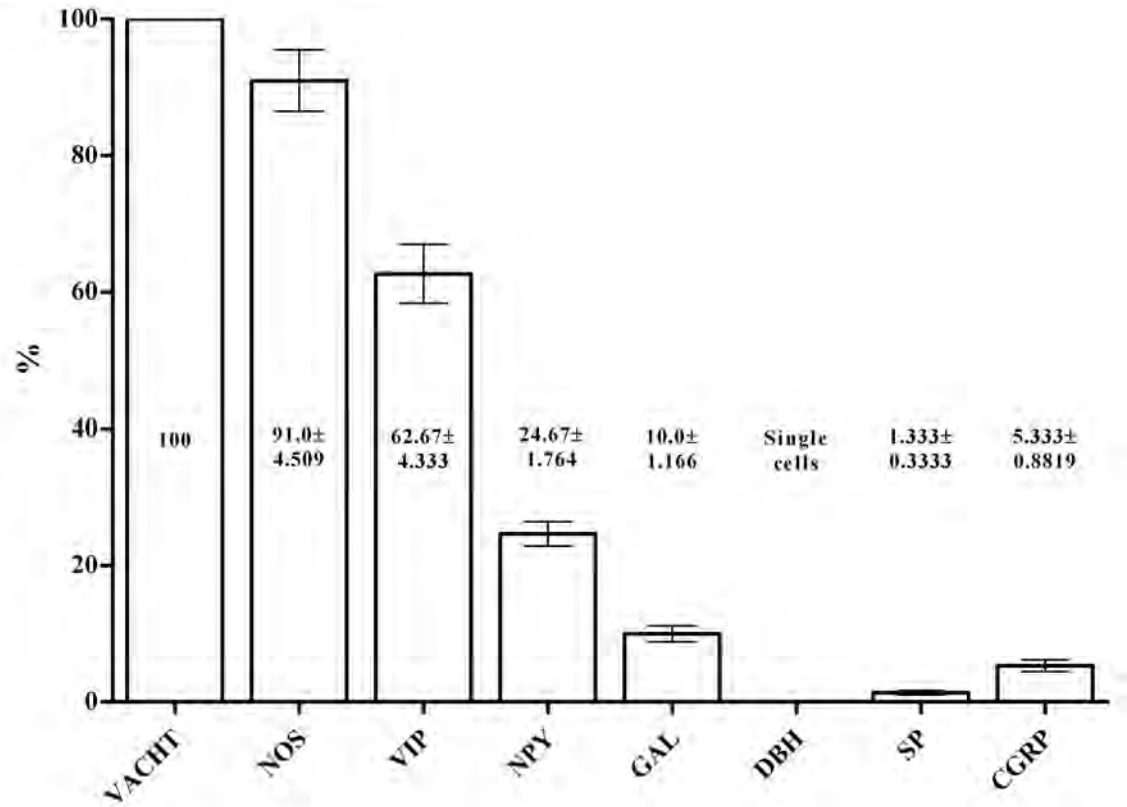

Fig. 7. Percentages of cholinergic (VAChT+ or ChAT+), adrenergic [dopamine β hydroxylase (DβH)+], NOS+, VIP+, NPY+, GAL+, CGRP+ and SP+ neurons among all PIL neurons. Bars and error bars represent means and SEM, respectively. Numerical data are given within or above the bars.

Table 2. Chemical coding of nerve fibres associated with neurons in porcine intrinsic laryngeal ganglia, and semiquantitative evaluation of their relative frequency.

	VACHT	NOS	VIP	NPY	GAL	SOM	DBH	SP	CGRP	Leu-Enk
Nerve fibres	++++	+	+	+	+	+	-	+++	++++	+

++++ – very numerous nerve fibres
+++ – numerous nerve fibres
++ – moderate number of nerve fibres
+ – small number of nerve fibres
± – single nerve fibers
- no nerve fibers

Immunoreactivity to NOS, GAL, VIP, SOM and Leu-Enk was found in small numbers of intraganglionic, varicose nerve fibres, which sometimes closely apposed the neuronal somata (Fig. 6).

Discussion

The present study has revealed for the first time the chemical coding of neurons and nerve fibres in porcine intramural laryngeal ganglia. It has also provided further evidence confirming earlier presumptions and observations that laryngeal intramural neuron-like cells are truly neurons.

The results of immunohistochemical investigations suggest that porcine laryngeal intramural neurons (ganglia) should be classified as cholinergic autonomic nerve structures. First, virtually all the nerve cells displayed immunoreactivities to cholinergic markers, ChAT and VAChT. Moreover, most of them also stained for other biologically active substances (NOS, VIP) commonly considered as typical of cholinergic neurons in many mammalian species. The present study has revealed only very solitary, small, intensely stained, DβH-positive ganglionic cells. In our opinion, they presumably represent interneurons (SIF, small intensely fluorescent, cells). Similar cells are found e.g. in parasympathetic intramural cardiac ganglia of many mammals including humans (Baptista and Kirby 1997).

The cholinergic nature of the vast majority of intralaryngeal neurons has been also found in all other species investigated (for review, see Hisa et al. 1999, 2016). In the rat, immunoreactivity to ChAT has been revealed in many intralaryngeal neurons (Nakanishi et al. 1999, Koike et al. 2004, Okano et al. 2006). Okano et al. (2006) have performed quantitative analysis and reported that all the neurons encountered contained immunoreactivity to this marker. Nakanishi et al. (1999) have applied double-staining for ChAT immunohistochemistry and AChE enzyme histochemistry and found that all the ChAT-positive somata revealed AChE activity. However, there were a few AChE-positive neurons that did not exhibit ChAT-immunoreactivity. Many intralaryngeal neurons in the cat are AChE-positive (Yoshida et al. 1993). However, some of them contain immunoreactivity to TH. It can be therefore assumed that in the cat, two populations of intralaryngeal neurons, significantly larger cholinergic and small adrenergic, are distinguished. As already mentioned, the present study has revealed only very solitary, small, intensely stained, DβH-positive intraganglionic cells that can be classified as presumably interneurons (SIF cells).

The present immunohistochemical investigations have disclosed that in addition to cholinergic markers, porcine laryngeal intramural neurons also contain NOS, VIP, NPY, GAL, SP, or CGRP. Percentages of the neuronal subpopulations suggest that some of the neurons express more than one of these substances (besides immunoreactivities to cholinergic markers).

It should be kept in mind that all the substances mentioned were expressed in the cholinergic neurons, thus they most probably functioned as neuromodulators or co-transmitters in relation to the main neurotransmitter, acetylcholine (ACh).

The presence of laryngeal intramural nitrergic neurons has been revealed in some other mammalian species with the use of NADPH-d histochemistry (Hisa et al. 1996a, b, c, Tsuda et al. 1998) and immunohistochemical stainings for NOS (Robertson et al. 1998, Koike et al. 2004).

In the cat, the majority of neuronal cells in the intralaryngeal ganglia have been found to be NADPH-d-positive (Tsuda et al. 1998). Clusters of NADPH-d-positive neuronal somata have been occasionally encountered in the rat and canine larynx (Hisa et al. 1996a, b, c). These authors have also observed some NADPH-d-positive nerve fibres supplying laryngeal blood vessels and submucosal glands. They have concluded that the nitrergic fibres originate from the intralaryngeal ganglia, and that NO is an endogenous modulator of cholinergic neurotransmission in the parasympathetic innervation for the regulation of blood flow and secretion in the larynx. It can be assumed that the cholinergic-nitrergic intramural laryngeal neurons perform similar functions in pigs.

VIP has been the most often localized neuropeptide in the mammalian intralaryngeal ganglia. Masuko et al. (1991) have revealed that in the dog, the vast majority, (nearly 97%) of intrinsic laryngeal neurons displayed immunoreactivity to VIP; of these 44% were immunoreactive to VIP alone, 22% to NPY, 13% to SP, 7% to Enk and 14% to VIP, NPY and SP. Koike and Hisa (1999) have found some VIP-positive intramural laryngeal neurons in this species. Immunoreactivity to VIP has been also determined in many (91%; Tsuda et al. 1992) feline intrinsic laryngeal neurons (Yoshida et al. 1993, Tanaka et al. 1993a) and in some rat local laryngeal neurons (Krekel et al 1990, Luts et al. 1990, Domeij et al. 1991a, b, Robertson et al. 1998, Koike et al. 2004). On the other hand, no VIP-immunoreactive neurons were observed in local ganglia of the equine larynx (Corcoran et al. 1999). It has been suggested that VIP-containing cholinergic intralaryngeal neurons are involved in vasodilation and reduction of seromucous secretion (Hisa et al. 1999).

NPY-positive neurons have been found in the intralaryngeal ganglia of rats (Luts et al. 1990, Domeij et al. 1991a, b) dogs (Masuko et al. 1991, Yamamoto et al. 1998), cats (Tsuda et al. 1992, Tanaka et al. 1993a, Yoshida et al. 1993) and humans (Luts et al. 1993) but their number is small compared with the number of VIP- or NOS-immunoreactive nerve cells. The involvement of the local cholinergic NPY-expressing neurons in the regulation of laryngeal exocrine secretion and blood flow seems not to be significant (Hisa et al. 1999).

The present study has revealed that in the pig, the GAL-containing neurons constitute a small but still significant population of the nerve cells. There is a paucity of studies reporting the presence of GAL-immunoreactive neurons in the mammalian intralaryngeal ganglia. Luts et al. 1993 have only mentioned the presence of a few nerve cell bodies coexpressing GAL and VIP in local ganglia of the human tracheal wall. Cheung et al. (1985) observed a few GAL-immunoreactive ganglion cells also containing VIP in the adventitia of the tracheobronchial wall in the pig and dog. The occurrence of GAL-containing neurons was also reported in the deep region of the submucosal layer in the canine glottis (Yamamoto et al. 1998). Unfortunately, these papers provide no detailed information on the number of the neurons, nevertheless, a careful analysis of these publications suggests that the population of GAL--expressing neurons in local ganglia associated with human, pig and dog airways is rather small, which is in contrast to our results.

It is difficult to speculate about the possible role of GAL expressed in intramural cholinergic neurons of the porcine larynx because the relevant literature data are sparse and even contradictory. The accumulated fragmentary information suggests that the local galaninergic neurons most probably participate in regulating airway responses mediated by sensory reflexes. First, GAL has no direct effect on airway smooth muscle tone (Giuliani et al. 1989), blood flow (Salonen et al. 1988), nor does it modulate smooth muscle contractions evoked by ACh (Conroy et al. 1991). Moreover, GAL has been found to not affect cholinergic bronchoconstrictor reflexes (Takahashi et al. 1994). However, GAL inhibits non-cholinergic contractions induced by field stimulation, which suggests a neuromodulatory action (Giuliani et al. 1989). Because non-cholinergic contractions are considered to be mediated by tachykinins released from sensory nerve terminals (Lundberg et al. 1983), the prejunctional sensory neuromodulation may be mediated through the release of GAL from axons originating in the local laryngeal ganglia.

The present study has revealed that a small number of the porcine laryngeal neurons stained for SP. The occurrence of the few SP-immunoreactive nerve cells has been also reported in dogs (Masuko et al. 1991) and cats (Tsuda et al. 1992, Tanaka et al. 1993b, Yoshida et al. 1993), however, such neurons have not been found in rats (Hisa et al. 1999) and humans (Frigo et al. 1989). Substance P-positive nerve fibres are distributed throughout laryngeal structures in many mammalian species including humans (for review, see Hisa et al. 1999). In rats most of these axons are thought to be of extrinsic origin and derive from sensory ganglia of the vagus nerve (Domeji et al. 1991c). However, in dogs (and, considering the present results, possibly also in pigs) some of them arise also from the local laryngeal ganglia and these nerve terminals are believed to be involved in the regulation of laryngeal blood flow and secretion (Masuko et al. 1991).

The expression of CGRP in a relatively substantial population (5.3%) of the porcine laryngeal neurons found in this study is an interesting finding, since such neurons have not been determined in rats (Domeji et al. 1991c), cats (Tsuda et al. 1992) nor dogs (Masuko et al. 1991).

Calcitonin gene-related peptide and SP are thought to be main neurotransmitters of the afferent system in laryngeal innervation (Hisa et al. 1994, for review, see Hisa et al. 1999, Hisa 2016). The distribution of the CGRP-positive nerve fibres in laryngeal structures suggests that they participate in the sensory responses, regulation of blood follow, glandular secretion and the motor function (Hisa et al. 1992, for review, see Hisa et al. 1999). Because, as mentioned, no CGRP-expressing intramural laryngeal neurons were found in earlier studies, CGRP-containing nerve fibers in the larynx were considered to be of extrinsic origin and some find-

ings suggested that they originated from the nodose ganglion (Cadieux et al. 1986, Terenghi 1986, Tsuda et al. 1992). However, our results imply that in the pig, part of them may also derive from the intralaryngeal ganglia.

When speculating about the functional significance of the intramural laryngeal cholinergic expressing CGRP or SP neurons it is worth mentioning that a number of postganglionic sympathetic cholinergic neurons innervating exocrine sweat glands in the skin contain these peptides (for review, see Lindh and Hökfelt 1990). Thus, it is tempting to assume that in the pig, the neurons in question may be involved in the regulation of the secretory function of various laryngeal glands. However, their participation in the control of the blood flow (especially the vasodilatory role) is even much more likely (for review, see Wehrwein et al. 2016).

It should be also mentioned that CGRP is widely expressed in cholinergic neurons in spinal and cranial somatic motor nuclei in the pig (Calka et al. 2009) and other mammalian species (Gibson et al. 1984, Moore 1989, for review, see Changeux et al. 1992).

The present study has revealed two major subpopulations of varicose nerve fibres associated with the intrinsic laryngeal neurons: those which stained for cholinergic markers (especially VAChT) and those which expressed SP and/or CGRP.

The literature in the field contains some information strongly suggesting that the cholinergic fibres can represent preganglionic axons. At first, there is a wide agreement that both sympathetic and parasympathetic preganglionic nerve terminals are cholinergic in nature (for review, see Wehrwein et al. 2016). Furthermore, the VAChT-positive nerve fibres found in this study supplied nearly all the neuronal somata, which is another observation confirming their preganglionic character.

With regard to the SP and/or CGRP-immunoreactive nerve fibres, it can be assumed they represent collaterals of primary sensory neurons. It is now generally accepted that primary sensory neurons transmit not only sensory information but also can release biologically active substances, mostly CGRP and SP, at the peripheral terminations of their processes, thereby affecting target organ functions (for review, see Maggi 1995). Moreover, these sensory fibres provide synaptic collaterals to neurons in autonomic ganglia, thus forming the basis for "axon reflexes" that permit afferent modulation of autonomic neuron activity (Cuello 1983, Suzuki et al. 1989, Parr et al. 1993). Taking into account what was previously mentioned about the origin of the sensory nerve fibers in the structures of the larynx it can be reasonably supposed that sensory collaterals in the human and porcine laryngeal intramural ganglia are processes of peripheral axons of neurons found in the sensory ganglia of the vagus nerve. This assumption is supported by the results obtained by Frigo et al. (1989) who have found that neurons in human ganglia located in the deep connective tissue of the laryngeal mucosa were devoid of SP-like immunoreactivity but were closely apposed by many SP-like immunoreactive varicose nerve fibers.

When discussing the non-classical neuronal communications (the interaction of sensory neurons via their collaterals on peripheral autonomic nerve cells is sometimes referred to in this way; Cuello 1983) it seems worth mentioning the possible interaction between the local laryngeal neurons and neurons in the so-called lingual enteric nervous system (LENS), which is believed to act as an initial chemical detector that analyzes the food before the ingestion and transmits this information distally (for review, see Sbarbati and Osculati 2007). It is considered that the connections between both systems can be important in terms of integration of afferent information from different receptor areas of the mucosa relevant to the deglutition/airway protection and they may be involved in airway reflexes after stimulation of the oropharyngeal and laryngeal mucosa. Therefore, it can be assumed that the SP- and/or CGRP-positive nerve fibres found in this study, as possible sensory neuron collaterals, play some role in the aforementioned dependencies.

In conclusion, this study has revealed that intrinsic neurons in the porcine larynx, like in many other mammalian species studied, should be classified as parasympathetic cholinergic neurons expressing biologically active substances, predominantly NOS and VIP. Furthermore, they are likely to receive inputs from not only preganglionic neurons but also from primary sensory nerve cells. Given the relatively large number of the neurons and the fact that they form, in addition to some smaller clusters, also a larger ganglion, it seems that the occurrence of the local laryngeal ganglia should be mentioned regularly in textbooks dealing with the cranial portion of the parasympathetic nervous system in mammals.

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