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Original article

Immunohistochemical characterization of neurons and neuronal processes in the dorsal vagal nucleus of the pig

M. Gańko¹, A. Rychlik², J. Całka¹¹ Department of Clinical Physiology, Faculty of Veterinary Medicine, Oczapowskiego 13, 10-718 Olsztyn, Poland² Department of Clinical Diagnostics, Faculty of Veterinary Medicine, University of Warmia and Mazury, Oczapowskiego 14, 10-719 Olsztyn, Poland

Abstract

The vagus nerve is responsible for efferent and afferent innervation of the gastrointestinal tract. The efferent fibres originate from neurons located in the dorsal motor nucleus of the vagus (DMX) and control mainly the gastric motor and secretory function.

The main goal of our study was to examine expression of ChAT, SP, LENK, NOS and CART in the neuronal matrix of the porcine DMX.

Double-labeling immunofluorescence revealed plenty of ChAT-IR neuronal cell bodies and fibres distributed throughout the nuclear matrix. Between the cholinergic somata and processes numerous SP-, LENK- and CART-IR neuronal protrusions forming dense networks were identified. While net of the NOS-IR fibres presented moderate density, the SP- and LENK-IR processes were often observed to form a basket-like structure closely surrounding the cholinergic parasympathetic neurons. Individual CART-IR basket-like formations were also encountered. A few double labeled ChAT-/NOS-IR perikarya in the rostral segment of the nucleus were also found.

We confirm expression of studied antigens in the porcine DMX and provide morphological foundations for a possible regulatory role of SP, LENK, NOS and CART in porcine vago-visceral signaling.

Key words: immunofluorescence, parasympathetic motor vagal neurons, SP, LENK, NOS, CART, swine.

Introduction

The vagus nerve constitutes the main pathway providing both afferent and efferent parasympathetic nerve supply for thoracic and abdominal cavity organs. Previous studies have demonstrated that vagal efferent fibres originate from two brainstem regions: the dorsal motor nucleus of the vagus (DMX) and the

nucleus ambiguus (NA). Neurons projecting to the upper gastrointestinal tract including the stomach, were localized in the DMX, whereas those located in the NA projected to the oesophagus and the cardiorespiratory system (Gwyn et al. 1985, Ewart et al. 1988, Yoshida et al. 1989, Chang et al. 2003, Ruggiero et al. 2004, Ammori et al. 2008). The preganglionic parasympathetic cells of the DMX not only integrate

peripheral and central signals concerning gastrointestinal processes but also directly influence gastrointestinal activities (Armstrong and Hopkins 1998, Palkovits 1999, Zhou et al. 2008, Ammori et al. 2008).

The DMX parasympathetic neurons exploit acetylcholine as a main neurotransmitter, as confirmed by expression of a specific cholinergic neuronal marker – choline acetyltransferase (ChAT) (Zalecki et al. 2007). Previous immunocytochemical analyses revealed the nuclear matrix as the place of expression of bioactive substances, possibly affecting integration and transduction of the efferent preganglionic signals (Blessing et al. 1986, Huang et al. 1993a, Perez et al. 2001).

It has been well established that substance P (SP) is contained in the dorsal vagal somata of the ferret (Boissonade et al. 1996) and human (Huang et al. 1993b, Fodor et al. 1994) as well as processes of the rat (Ladic and Buchan 1996), pigeon (Berk et al. 1993) and cat (Baude et al. 1992). Additional evidence that SP-IR processes of the DMX oppose the stomach supplying the DMX perikarya constitutes morphological proof for functional interactions between both neural structures (Ladic and Buchan 1996). However, to our knowledge, the histochemical characteristic of vagal preganglionic cell bodies as well as neighboring processes in the pig remains completely unknown.

In view of former reports it has been quite clear that enkephalins contribute to peptidergic innervation of the rat (Khachaturian et al. 1983), dog (Pego-Reigosa et al. 2000), ferret (Boissonade et al. 1996) and human (Covenas et al. 2004) DMX. Although opioid peptides, in particular enkephalins, play a crucial role in gastrointestinal regulations (Browning et al. 2002) there is still a lack of anatomical data regarding organization of the LENK-immunoreactive system in the DMX of the pig.

Both histochemical as well as immunocytochemical mapping revealed that neurons of the rat DMX may synthesize nitric oxide (NO) (Vincent and Kimura 1992, Rodrigo et al. 1994). Further studies disclosed nitrergic neurons in the DMX of other species including the cat and dog (Panico et al. 1995, Maisky et al. 2003) and primate (Lin et al. 2000). Although NO releasing neurons have been shown to be involved in many physiological processes including food intake and drinking (Bruhwyler et al. 1993), excitation of the extrahepatic biliary system (Jinyan et al. 2001), stimulation of gastric contraction and relaxation (Zhou et al. 2008) or neuronal reaction to injury (Zhao et al. 1996, Lin et al. 1997), the identity of nitrergic nerve structures in the porcine DMX has not yet been confirmed.

Hitherto, CART immunoreactive (CART-IR)

perikarya and processes have been reported in the DMX of the immature rat (Dun et al. 2001); however, other investigators were not able to identify CART-IR structures in the nucleus of mature animals (Koçlu et al. 1998, Broberger et al. 1999). Although the functional importance of CART has been studied for three decades there is a scarcity of data concerning distribution and possible significance of CART-IR structures in the DMX of other species, especially the swine, relevant to biomedical research (Verma et al. 2011).

The pig, due to its anatomical and physiological similarities to the human, is generally used as an experimental model for bio-medical research (Swindle et al. 1992, Verma et al. 2011). Therefore, the aim of our investigation was to analyze the localization and distribution of SP, LENK, NOS and CART in the dorsal motor nucleus of the vagus of the pig. Additionally, the spatial correlation between the cholinergic preganglionic vagal perikarya and the peptidergic nerve cell protrusions was studied.

Materials and Methods

The experiments were carried out on five immature gilts (about 20 kg of body weight) of the Large White Polish breed. All animals were kept under standardized conditions with free access to water and food appropriate for age. The animals were housed and treated in accordance with the rules approved by the local Ethics Commission.

All gilts were pre-treated with azaperone (Stresnil, Jansen Pharmaceutica N.V., Belgium; 2 mg/1 kg of body weight given intramuscularly) 15 min before application of the main anesthetic, sodium thiopental (Thiopental, Sandoz, Kundl-Rakusko, Austria; 20 mg/kg of body weight given intravenously) and were euthanized by an overdose of sodium thiopental and then perfused transcardially with 4% buffered paraformaldehyde (pH 7.4) prepared *ex tempore*. Medullas for further research were collected from all studied animals.

Samples were then postfixed by immersion in the same fixative for 20 min, washed with 0.1 M PB (pH 7.4) over three days and finally transferred to 30% buffered sucrose solution (pH 7.4), containing 0.01% sodium azide and stored at 4°C.

Frozen samples were cut in a cryostat into 14 µm thick sections, mounted on chrome alum-coated slides and processed for immunocytochemistry, applying a routine double-labelling immunofluorescence technique.

Briefly, after air-drying at room temperature for 45 min and rinsing in 0.1 M phosphate-buffered saline

Table 1. Description of antibodies.

Antigen	Species	Code	Manufacturer/Supplier
Primary antibodies			
ChAT	goat	AB144P-1ML	Millipore, USA
SP	rat	T-1609	Bachem, Switzerland
LENK	rabbit	EA 1149	BIOMOL, USA
NOS	rabbit	AB5380	Chemicon, USA
CART	rabbit	H-003-61	Phoenix Pharmaceuticals, USA
Secondary antibodies			
Alexa Fluor 488 nm anti-goat		A11055	
Alexa Fluor 546 nm anti-rat		A11081	Invitrogen, USA
Alexa Fluor 546 nm anti-rabbit		A10040	

(PBS; pH 7.4; 3 x 10 min), the sections were incubated in a blocking buffer containing: 0.1% BSA (bovine serum albumin) in 0.1 M PBS, 1% Triton X-100, 0.05% Thimerosal, 0.01% sodium azide for 1 h at room temperature to reduce non-specific background staining. Subsequently, after a second wash in PBS (3 x 10 min), the sections were incubated overnight at room temperature with a mixture (various combinations: ChAT/SP; ChAT/LENK; ChAT/nNOS; ChAT/CART) of goat polyclonal anti-ChAT antibody in a working dilution of 1:50 and/or rat anti-SP antibody in a working dilution of 1:150, rabbit anti-LENK antibody in a working dilution of 1:5000, rabbit anti-nNOS antibody in a working dilution of 1:6000, rabbit anti-CART antibody in a working dilution of 1:10000 (Table 1).

Following subsequent rinsing in PBS (3 x 10 min), the sections were incubated at room temperature for 1 h with donkey anti-goat (Alexa Fluor 488 nm, green) and a mixture of donkey anti-rabbit (Alexa Fluor 546 nm, red) and goat anti-rat (Alexa Fluor 546 nm, red) fluorescent antibodies in a dilution of 1:1000 (Table 1).

Finally, the slides were rinsed in PBS (3x10 min) and then coverslipped with carbonate-buffered glycerol (pH 8.6).

The omission of the primary antisera as well as their replacement with normal sera proved the specificity of the immunoreaction. The methodology of the preabsorption control has been described by Arciszewski et al. (2008).

The slides were then analysed under an Olympus BX51 microscope equipped with epi-fluorescence and appropriate filter sets and photographed.

Results

In the studied medulla a noticeable concentration of ChAT-immunoreactive perikarya, located in its dorsolateral area, forming the dorsal vagal motor nucleus, was found. Light microscopic analysis revealed its cytoarchitecture to be congruent to that previously reported (Zalecki et al. 2007). The nuclear group disclosed a heterogeneous structure depending on the cross section level. Between its rostral and caudal compact groups, the middle nuclear part divided into two subdivisions, forming a semilunar shape, was observed. The majority of the perikarya were oval, round or multipolar in shape with a centrally situated nucleus. The cell bodies measured about 20 to 40 μ m in diameter. A large number of the ChAT-immunoreactive fibers were dispersed between ChAT-positive cells.

Microscopic examination showed an intense network of SP-IR nerve fibres throughout the nuclear region. Varicose nerve processes often encircled ChAT-positive cell bodies, enabling direct contacts between adjacent structures (Fig. 1a,b,c).

Application of anti-LENK antibody revealed a dense network of LENK-positive nerve processes running between cholinergic perikarya. They were composed of thick varicosities and thin intervaricose segments. The processes formed many basket-like structures surrounding ChAT-IR neurons (Fig. 2a,b,c).

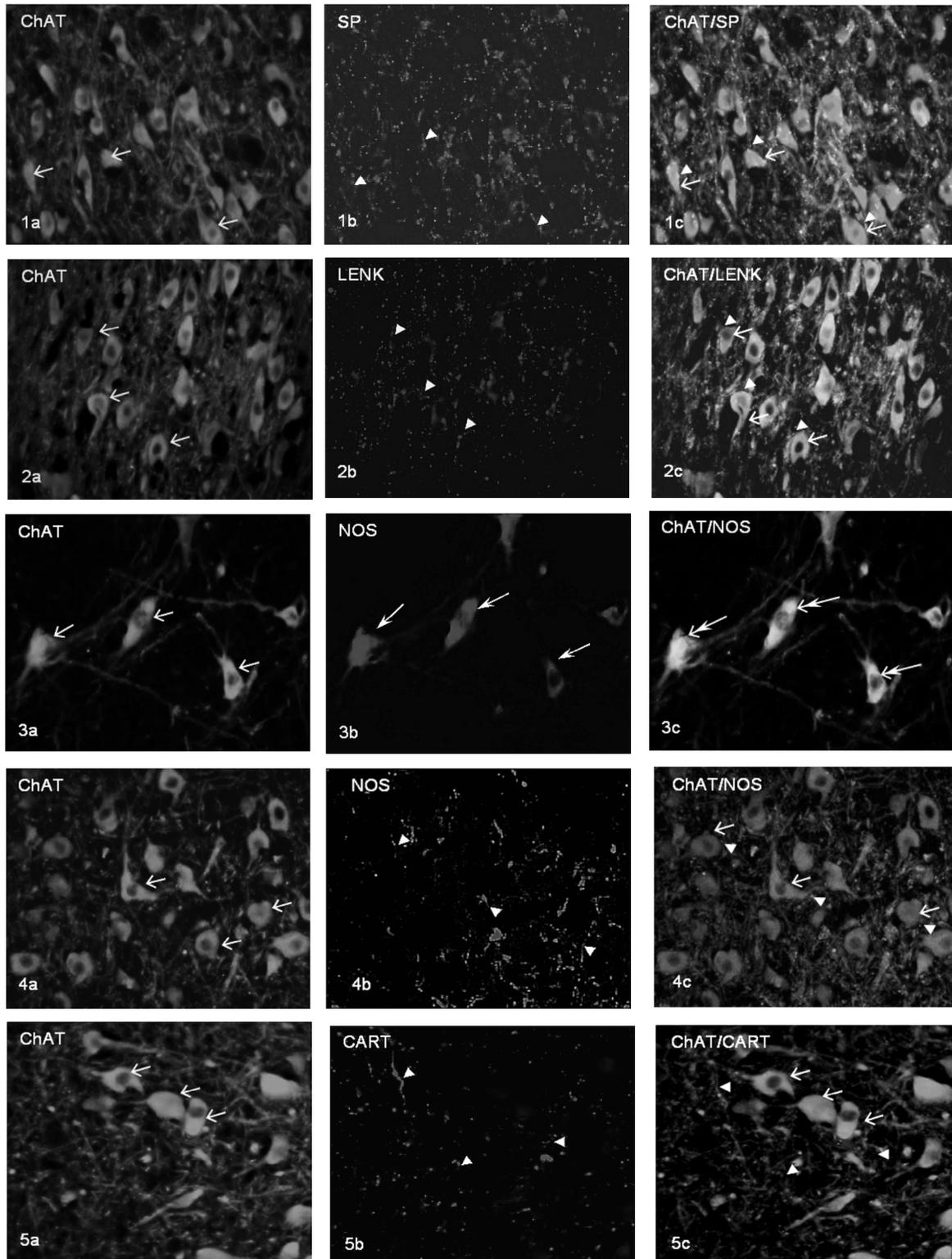


Fig. 1. a) ChAT-IR neurons (arrows) in porcine DMX; b) SP-IR fibres (arrow heads) in DMX; c) SP-IR fibres (arrow heads) encircle ChAT-positive cell bodies (arrows) enabling direct contacts; Fig. 2. a) ChAT-IR neurons (arrows) in DMX; b) LENK-IR fibres (arrow heads) in DMX; c) LENK-IR fibres (arrow heads) form basket-like structures surrounding ChAT-IR neurons (arrows); Fig. 3. a) ChAT-IR neurons (small arrows) in DMX; b) NOS-IR neurons (large arrows) in DMX; c) Double labeled ChAT-/NOS-IR neurons (double arrows) in DMX; Fig. 4. a) ChAT-IR neurons (arrows) in DMX; b) NOS-IR fibres (arrow heads) in DMX; c) NOS-IR fibres (arrow heads) penetrating intercellular matrix between ChAT-IR perikarya (arrows); Fig. 5. a) ChAT-IR neurons (arrows) in DMX; b) CART-IR fibres (arrow heads) in DMX; c) CART-IR fibres (arrow heads) penetrating intercellular matrix between ChAT-IR perikarya (arrows). Photographs 1c, 2c, 3c, 4c, 5c were prepared by superimposition of single stainings. Magnification in Figs. 1, 2, 4, 5 x 200 and in Fig. 3 x 400.

Immunofluorescent analysis revealed local coexpression of the NOS in cholinergic neurons disclosed at the very rostral edge of the DMX. The cellular cluster was composed of a few double labeled perikarya (Fig. 3a,b,c). NOS-positive delicate fibers occupied the intercellular matrix throughout the entire rostro-caudal extent of the nucleus. Although they ran closed to ChAT-IR cell bodies their close spatial relationship was rarely observed (Fig. 4a,b,c).

Double-immunostaining of the medullary sections revealed separate expression of ChAT and CART. CART-immunoreactivity was restricted to tiny nerve processes penetrating the intercellular matrix. Although direct apposition between the protrusions and cholinergic cell bodies were only occasionally encountered, single cholinergic perikarya encircled by visible CART-IR fibres were noticed (Fig. 5a,b,c).

Discussion

This study provides data on the distribution of SP, LENK, NOS and CART in the dorsal vagal motor nucleus of the pig. The results show a dense network of SP-, LENK-, NOS- and CART-IR nerve processes crossing the nuclear matrix between ChAT-IR parasympathetic perikarya, thus providing morphological evidence for a possible direct interaction between visualized neural protrusions and the nuclear cell bodies. Our examination revealed that SP- and LENK -IR processes apposed cholinergic cell bodies, often forming basket-like structures closely surrounding the ChAT-IR somata. This particular spatial relationship was only occasionally observed between cholinergic perikarya and CART-IR processes. On the other hand, NOS-labeled fibers seemed to avoid establishing direct contact with the nuclear neurons. These anatomical discrepancies may reflect differential regulatory action on vagal cholinergic signaling.

Our discovery of a dense network of SP-IR fibers running in close proximity to the porcine DMX neurons provides a morphological foundation for direct regulatory action of SP expressing processes on preganglionic vagal somata. Taking into account the fact that these preganglionic neurons project to the gastric regions, the proximal duodenum (Berthoud et al. 1991) and to the greater curvature (Ladic and Buchan 1996) SP released from the SP-ergic processes might affect gastrointestinal function. Indeed, Ladic and Buchan (1996) identified SP-IR processes in close association with gastric DMX neurons in the rat, thus providing evidence for their direct mutual interaction. The biological significance of this anatomical correlation has been demonstrated by the finding that rat vagal neurons in the DMX have NK₁ receptors for SP

(Plata-Salaman et al. 1989, Dixon et al. 1998, Lewis and Travagli 2001) and SP in the rat DMX inhibits gastric acid secretion (Yang and Tache 1997), evokes feline pylorus contraction (Edin et al. 1980) and rat gastric relaxation (Spencer and Talman 1986). Since the spatial relationship of both SP-IR processes and efferent vagal somata in the porcine DMX closely resembles those in laboratory animals, the SP-ergic processes in the porcine DMX as in small animals, may affect gastrointestinal function. Nevertheless, further physiological studies confirming this phenomenon in the pig are necessary.

Our examination revealed that porcine cholinergic neurons in DMX are closely surrounded by LENK-IR nerve fibres. The presence of LENK-IR processes in the porcine DMX is consistent with previous findings in the rat, which described the LENK-IR terminal projections to the dorsal vagal complex from the paratrigeminal nucleus (Armstrong et al. 1998). Evidence indicating the involvement of opioid transmission to DMX, as a component of the dorsal vagal complex, originating from neurons of the NTS, amygdala or periaqueductal gray (Morilak et al. 1989, Pickel et al. 1989, Maley 1996, Farkas et al. 1997, Giraudo et al. 1998, Liubashina et al. 2000) additionally support the functional significance of LENK related transmission in porcine DMX. In fact, activation of central opioid receptors have been shown to induce gastric relaxation (Burks et al. 1987, Gue et al. 1989), decrease gastric acid secretion (Del Tacca et al. 1987) and increase feeding (Giraudo et al. 1998). Identification of the close spatial relationship between LENK-IR processes and the preganglionic parasympathetic cell bodies shed light on its possible regulatory function in the porcine DMX.

Immunofluorescent detection of the neuronal NOS revealed local coexpression of the NOS in cholinergic neurons, disclosed at the very rostral edge of the porcine DMX, as well as a network of delicate nitrergic processes intermingled between cholinergic somata. This dual expression pattern is consistent with that observed in other species (Vincent and Kimura 1992, Rodrigo et al. 1994, Panico et al. 1995, Lin et al. 2000, Maisky et al. 2003) and provides a morphological foundation for both a direct and indirect NO dependent local regulatory mechanism. Since retrograde (Leslie et al. 1990) and anterograde (Berthoud et al. 1991) neural tracers disclosed that around 80% of DMX neurons have projections to the stomach, NO released from the double labeled cholinergic/NO-ergic preganglionic neurons might directly affect gastrointestinal function, while NO released from processes might exert a local regulatory action restricted to the nuclear area. It is worth mentioning that, in the rat, cholinergic preganglionic vagal neurons constitute

heterogeneous population composed of ChAT-IR/ /NOS-IR somata as well as ChAT-IR/ TH-IR but NOS negative cells (Guo et al. 2001). Keeping in mind that NO is not released into the synaptic space and does not act at the postsynaptic membrane, but diffuses through cell membranes to bind to the iron of the heme moiety of soluble guanylyl cyclase or cyclooxygenase in its targets cells (Grossman et al. 1997, Goretski and Hollocher 1998, Calka 2006), thus using cyclic GMP and prostanoids as second messengers, and its high permeability range up to 300 μm (Garthwaite and Boulton 1995) NO released by both neural somata and processes may affect simultaneously different neuronal formations from the DMX regulatory level. This unique regulatory mechanism of NO-ergic transmission suggests that NO-immunoreactive perikarya and fibers localized in the porcine DMX may affect multiple regulatory vagal processes.

Immunocytochemical data in this study indicate the occurrence of tiny CART-IR nerve processes, while no positive cell bodies were encountered in the DMX of the juvenile pigs. Our findings concerning visualization of the processes are generally consistent with those reported in the adult and postnatal rat (Dun et al. 2001, Zheng et al. 2002, Jelsing et al. 2008). However, CART-IR somata have been identified in mature rat DMX (Zheng et al. 2002, Jelsing et al. 2008). Developmental studies (Dun et al. 2001) revealed that CART expression in the rat DMX neuronal somata undergoes reduction during developmental changes, such that few neurons appear to contain CART immunoreactivity in mature rats. Although absence of CART-IR perikarya in the porcine DMX might reflect interspecies differences in local CART expression, it seems more probable that future application of more sensitive visualization methods e.g. *in situ* hybridization will confirm CART expression in the porcine tissue. On the other hand, the multi-source origin of the CART-IR processes innervating the rat DMX, such as vagal afferent, medullary reticular formation, arcuate/retrochiasmatic nucleus of the hypothalamus, and likely area postrema (Zheng et al. 2002) may reflect the significant position of the CART-IR processes in vagal inhibition of gastric acid secretion (Okumura et al. 2000) as well as CART induced suppression of gastric emptying (Smedh and Moran 2006). Since the present work represents the first attempt to visualize CART-IR nerve formations in the porcine DMX further morphological as well as functional studies are required to elucidate its exact structure and physiological significance in this species.

Our study provides novel data on the expression of ChAT, SP, LENK, NOS and CART in the porcine

DMX and in particular provides detail on the spatial relationship between cholinergic preganglionic vagal neurons and peptidergic nerve processes expressing SP, LENK, NOS and CART in the nucleus. We report on the especially close apposition of the SP- and LENK-immunoreactive processes with cholinergic vagal neurons, thus providing morphological ground for direct vago-visceral signaling in pig, the most valuable species in biomedical research.

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