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Original article

Immunohistochemical properties of motoneurons supplying the porcine trapezius muscle

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Abstract

The trapezius muscle (TRAP) belongs to the scapulothoracic group of muscles, which play a crucial role in the integrity and strength of the upper limb, trunk, head, and neck movements and, thus, in maintaining balance. Combined retrograde tracing (using fluorescent tracer Fast Blue, FB) and double-labelling immunohistochemistry were applied to investigate the chemical coding of motoneurons projecting to the porcine TRAP. FB-positive (FB+) motoneurons supplying the cervical (c-TRAP) and thoracic part (th-TRAP) of the right (injected with the tracer) TRAP were located within the IX-th Rexed lamina in the ipsilateral ventral horn of the grey matter of the spinal medulla. Immunohistochemistry revealed that nearly all the neurons were cholinergic in nature [choline acetyltransferase (CHAT)- or vesicular acetylcholine transporter (VACHT)-positive]. Many retrogradelly labelled neurons displayed also immunoreactivity to calcitonin gene-related peptide (CGRP; approximately 68% of FB+ neurons). The smaller number of nerve cells (5%, 3%, 2% or 1%, respectively) stained for nitric oxide synthase (n-NOS), vasoactive intestinal polypeptide (VIP), neuropeptide Y (NPY) and substance P (SP). The retrogradely labelled neurons were closely apposed by nerve fibres expressing immunoreactivity to CHAT, VACHT, CGRP, SP, DβH, VIP, n-NOS, NPY, GAL, Leu-Enk and Met-Enk. Taking into account the clinical relevance of TRAP, the present results may be useful in designing further research aimed at the management of various dysfunctions of the muscle.

Keywords: pig, trapezius muscle, tracing, motoneurons, immunohistochemistry

Introduction

The trapezius muscle (TRAP) is the most prominent landmark of the surface anatomy of the back-interscapular region. It belongs to the scapulothoracic group of muscles, which play a crucial role in the integrity and strength of the limb, trunk, head, and neck movements and, thus, in maintaining balance. The impaired motor innervation of the TRAP may cause painful and disabling conditions, such as significant muscle weakness or even paralysis (Wiater and Bigliani 1999, Chan and Hems 2006). Without proper management of this disorder, the shoulder girdle loses stabilization, which may eventually generate subluxation of the glenohumeral and sternoclavicular joints (Neumann 2017). On the other hand, disturbed motor control of the muscle may also result in its excessive activation which may lead to myofascial pain syndrome (MPS), the most common cause of chronic musculoskeletal pain (Akamatsu et al. 2015). In humans, especially performing long-lasting tasks requiring sustained TRAP activity (such as working on a computer), MPS often manifests as work-related trapezius myalgia (Meulemeester et al. 2017). Attenuated TRAP stimulation is characterized by pain, stiffness, and tightness of the muscle and the occurrence of hyperirritable points in certain muscular and fascial areas, known as myofascial trigger points, which when compressed, produce a referred pain (Akamatsu et al. 2015, Meulemeester et al. 2017). It has been found, that the location of these trigger points is strongly correlated with the position of motor end plates within the muscle (Chu 1995, Akamatsu et al. 2015). Undoubtedly, from the clinical perspective, detailed knowledge of motor muscle control is crucial for understanding the mechanisms involved both in maintaining its proper function and those underlying the pathological disturbances of its contractility.

The porcine TRAP is widely used as a model in studies dealing with the physiology and pathophysiology of skeletal muscles (Handel and Stickland 1986, Rock and Kozak-Reiss 1987, Iaizzo and Lehmann-Horn 1989, Rock et al. 1990, Lefaucheur 1990, Jones et al. 1999, Gardan et al. 2006, Lu et al. 2010, Arlotta et al. 2011). In pigs, TRAP is a broad, triangular-shaped muscle divided into two parts, the cervical (c-TRAP) and thoracic (th-TRAP), which play different but complementary roles in its function. The innervation of the porcine TRAP, especially its motor component, has a unique arrangement. The c-TRAP receives the motor input via the dorsal branch of the accessory nerve only, while th-TRAP, in addition to the dorsal branch, is also innervated by ventral branches of spinal nerves (Dudek et al. 2015). The localization of motoneurons supplying both parts of the TRAP has been described in detail in a previous paper, which additionally concerned the involvement of the dorsal branch of the accessory nerve (DBXI) in the muscle innervation (Dudek et al. 2015). The available literature contains no data on the immunohistochemical properties of motoneurons projecting to any specific skeletal muscle in the domestic pig, or humans, however, there is some information dealing with the general chemical coding of motoneurons located in ventral horns of the grey matter of the spinal cord in the pig (Merighi et al. 1990), rat (Piehl et al. 1993, Hisa et al. 1994, Fernandez et al. 2003, Atoji et al. 2005), horse (Merighi et al. 1990), cattle (Chiocchetti et al. 2005) and monkey (Ichikawa and Shimizu 1998).

The unique anatomical arrangement of motor innervation of c-TRAP in the pig (Dudek et al. 2015) suggests that in this species also other features of the innervation including the chemical coding of the motoneurons can be unusual. Therefore, it seems that detailed information on the organization of the motor innervation of the porcine TRAP including the distribution, nerve pathways, and chemical coding of neurons supplying both parts of this muscle would not only complete the missing knowledge but also create a solid base for further investigations dealing with the physiology and pathology of mammalian striated muscles. Finally, it should be also emphasized that the pig is not only a significant animal for veterinary medicine and agriculture but also it has become a critically important experimental animal model in biomedical research, due to much higher structural and functional resemblance between porcine and human organs and tissues compared to those in laboratory rodents (Swindle et al. 2012, Lunney et al. 2021, Hou et al. 2022).

Materials and Methods

The study was carried out on six juvenile female pigs of the Polish Landrace breed weighing 20-25 kg each. The animals were housed and treated following the rules approved by the local Ethics Commission (affiliated to the National Ethics Commission for Animal Experimentation, Polish Ministry of Science and Higher Education, permission no 29/2009). The pigs were divided into two groups (n=3), A and B. All the animals were pre-treated with propionylpromasine (Combelen, Bayer, Germany; 0.4 mg/kg of b.w., i.m.) 30 min. before the main anaesthetic drug, pentobarbital (Vetbutal, Biowet, Poland; 25 mg/kg of b.w.), was given intravenously. In group A, the animals were injected with the fluorescent retrograde tracer Fast blue (FB); 5% suspension of FB in distilled water) into the right c-TRAP; in group B, the animals were injected with FB into the right th-TRAP. The skin and subcuta-

Tab.1. Antisera used in the study.

Antigen	Host	Type	Dilution	Cat. No.	Lot/Batch	Supplier
Primary antisera						
CHAT	goat	polyclonal	1:50	AB144P	LV1480420	Milipore
VACHT	rabbit	polyclonal	1:4000	V5387	095K4751	Sigma
CGRP	rabbit	polyclonal	1:2000	11535	2659F	Cappel
NPY	rabbit	polyclonal	1:400	NA1233	Z07336	Biomol
n-NOS	rabbit	polyclonal	1:2000	11736	8648C	Cappel
n-NOS	mouse	monoclonal	1:100	N2280	081K4815	Sigma-Aldrich
VIP	rabbit	polyclonal	1:2000	Ab22736	552916	Abcam
VIP	mouse	monoclonal	1:500	MaVIP	91278	East Acres Biologicals
SP	rabbit	polyclonal	1:600	LSC42165	14326	Lifespan
SP	rat	monoclonal	1:150	8450-0505	NC134	ABD Serotec, UK
Gal	rabbit	polyclonal	1:2000	RIN7153	990921-2	Peninsula Lab.
Leu-Enk	rabbit	polyclonal	1:500	RPN1552	5	Amersham
Leu-Enk	mouse	monoclonal	1:500	4140-0355	21010811	ABD Serotec, UK
Met-Enk	rabbit	polyclonal	1:500	RPN1562	11461	Amersham
DßH	mouse	monoclonal	1:500	MAB308	0606031668	Milipore
PACAP	rabbit	polyclonal	1:5000	IHC8922	970737	Peninsula
Secondary antisera						
Host		Fluorochrom	Dilution	Code	Lot	Supplier
Donkey-anti-goat IgG (H+L)		Alexa fluor 546	1:500	A11056	399681	Invitrogen
Donkey-anti-rabbit IgG (H+L)		Alexa fluor 488	1:500	A21206	57542A	Invitrogen
Donkey-anti-mouse IgG (H+L)		Alexa fluor 488	1:500	A21202	536050	Invitrogen
Goat-anti-rabbit IgG (H+L)		Alexa fluor 488	1:500	A11008	51385A	Invitrogen
Goat-anti-mouse IgG (H+L)		Alexa fluor 488	1:500	A11001	632115	Invitrogen
Goat-anti rabbit IgG (H+L)		Alexa fluor 568	1:500	A11011	623962	Invitrogen
Goat-anti-mouse IgG (H+L)		Alexa fluor 568	1:500	A11004	49399A	Invitrogen
Goat-anti-rat IgG (H+L)		Alexa fluor 555	1:500	A21434	1670155	Invitrogen

neous tissue were cut over the corresponding part of the trapezius muscle that was exposed. (group A, cervical part, group B, thoracic part of the trapezius muscle). In each pig, the tracer was administered in 10 injections (4µl of FB each). The injection points were placed in a line along the muscle at its mid-height and were approximately 1 cm apart. Injections were made using a Hamilton syringe (10 µl, model 801), with a 26s gauge needle. After injection a portion of FB, the needle was held at the injection site for several dozen seconds to prevent leakage of the tracer from the injection site. The wounds were finally sutured and the surgical site was disinfected. After a survival period of 5 weeks, the pigs were deeply reanesthetized (following the same procedure as described above) and then, they were transcardially perfused with 0.5l of preperfusion solution containing 0.9% sodium chloride (Chemia, Gliwice, Poland), 2.5% polyvinylpyrolidone (Sigma, Deisenhofen, Germany), 0.5% procaine hydrochloride (Polfa, Warsaw, Poland), and 20 000 IU of heparin (Heparinum; Polfa; added extempore), followed by 8-10l of 4% ice-cold buffered paraformaldehyde (pH 7.4). No contamination with FB was found in tissues, muscles and skin, surrounding the places of the tracer injections. The following tissue blocks were collected: medulla oblongata (MO) and the cervical and thoracic parts of the spinal cord (SC). The tissues collected were then 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.

The MO and SC were cut into 20-µm-thick cryostat sections, mounted on glass slides and viewed under the fluorescent microscope equipped with a filter block for FB. FB-positive (FB+) neurons were counted in every fourth (medulla oblongata, spinal cord) section to avoid double analysis of the same nerve cell. The selected sections comprising FB+ neurons were processed for double immunofluorescence method using primary antisera against choline acetyltransferase (CHAT), vesicular acetylcholine transporter (VACHT), calcitonin gene-related peptide (CGRP), nitric oxide synthase

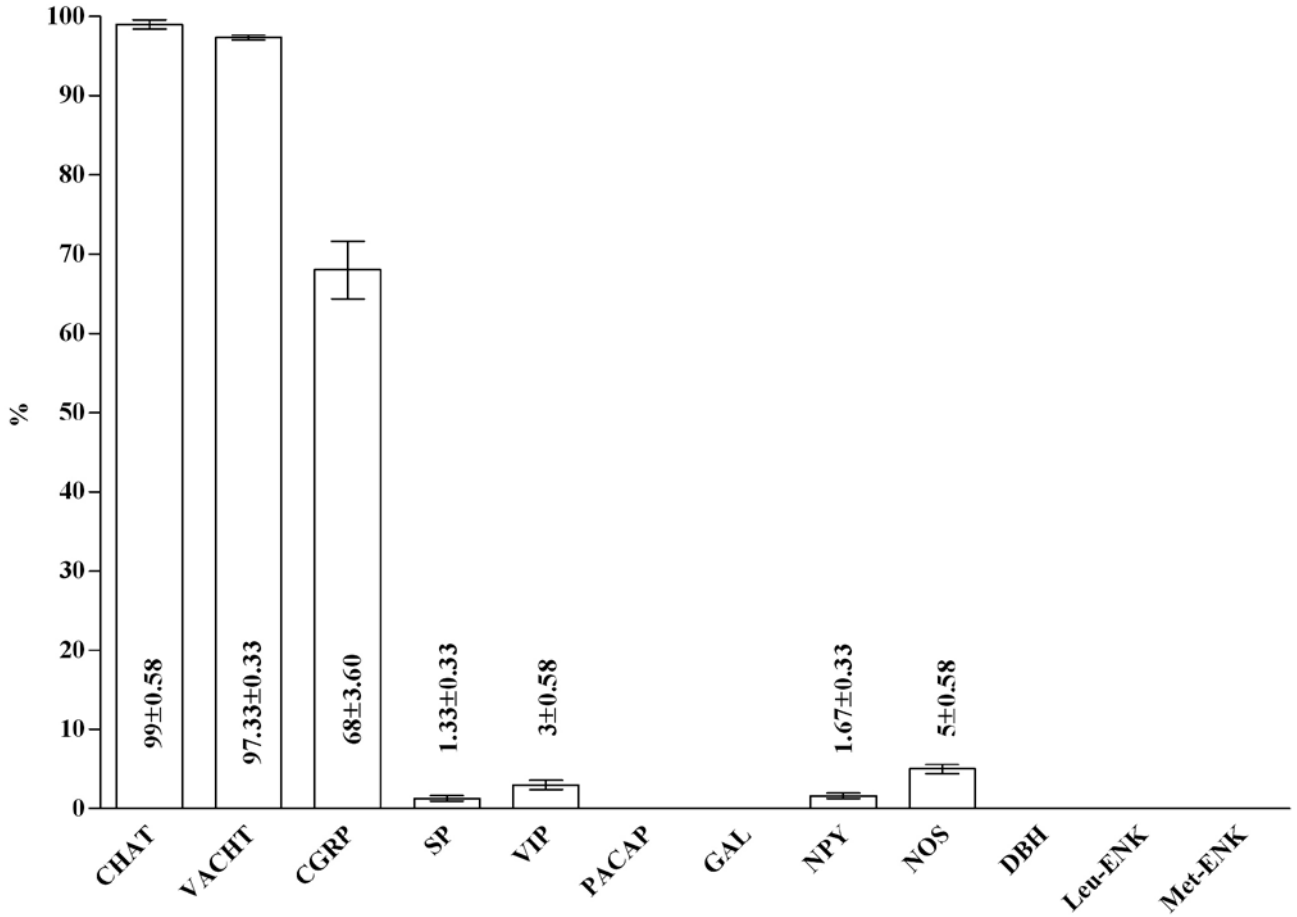

Fig. 1. Percentages of different neuronal populations among all Fast Blue-positive (FB+) motoneurons supplying the trapezius muscle in the pig. Bars and error bars represent means and SEM, respectively. Numerical data are given within or above the bars.

(n-NOS), vasoactive intestinal polypeptide (VIP), neuropeptide Y (NPY), substance P (SP), pituitary adenylate cyclase-activating peptide (PACAP), dopamine beta-hydroxylase (DβH), galanin (Gal) and enkephalins: Leu-5-nekphalin (Leu-Enk) and Met--enkephalin (Met-Enk) (Table 1) according to the method described previously (Wessendorf and Elde 1985, Dudek et al. 2011). Then they were investigated and images were recorded with 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.

To determine percentages of the particular neuronal subpopulations, at least 100 of FB+ neuronal profiles investigated with one combination of the primary antisera for the coexpression of the biologically active substances were counted in every animal. The percentages of the FB+ neurons immunopositive to the neuroactive substances were presented as mean ± SEM.

Standard controls, i.e. preabsorption for the neuropeptide antisera (20 μg appropriate antigen per 1 ml corresponding antibody at working dilution) or omission and replacement of the respective primary antiserum with the corresponding non-immune sera, completely abolished immunofluorescence and eliminated staining.

Results

As previously described (Dudek et al. 2015) FB+ neurons supplying the c-TRAP and thoracic th-TRAP were localized within the ipsilateral ventral horn of the grey matter of the first four and five cervical neuromeres, respectively. They were multipolar in shape with a relatively large diameter ranging from 80 to 120 μm, and were located within the IX-th Rexed lamina in the ipsilateral ventral horn of the grey matter of the spinal medulla, which corresponds with the localization of the spinal accessory nucleus (SAN).

Immunohistochemistry revealed that nearly all TRAP-projecting motoneurons were cholinergic in nature since 99±0.58% and 97±0.33% (Fig. 1) of them displayed CHAT (Figs. 2, 9) or VACHT-immunoreactivity, respectively (Figs. 3-8).

CHAT+ and CHAT+/CGRP+ neuronal somata were found to be surrounded by numerous, delicate, varicose CHAT-immunoreactive nerve fibers (Fig. 2). Moreover,

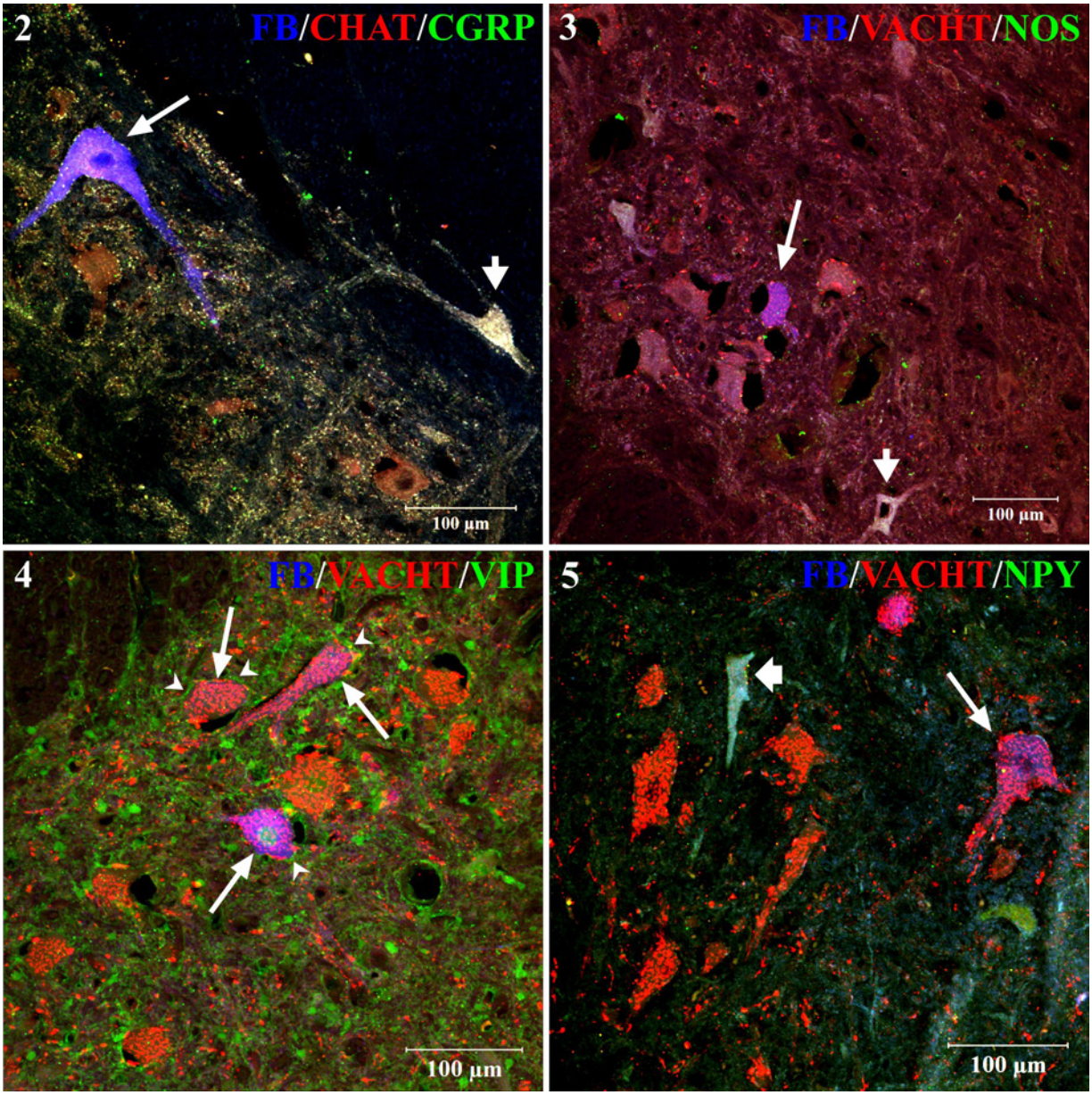

Fig. 2. Confocal laser scanning microscope image showing FB+ neurons in the second cervical spinal segment (neuromere C2) of the pig injected with FB into the cervical part of the trapezius muscle (c-TRAP) which were simultaneously choline acetyltransferase (CHAT)+ [red; Alexa 555 visualization (A555)] and calcitonin gene-related peptide (CGRP)+ [(green; Alexa 488 visualization (A488)] (short arrow) or stained for CHAT only (long arrow); blue, red and green channels were digitally superimposed (BRGDS).

Fig. 3. Confocal laser scanning microscope image showing FB+ motoneurons in the C3 of the pig injected with FB into c-TRAP which were simultaneously vesicular acetylcholine transporter (VACHT)+ (red; A555) and nitric oxide synthase (NOS)+ (green; A488) short arrow) or NOS-immunonegative (NOS-; long arrow); BRGDS.

Fig. 4. Confocal laser scanning microscope image showing FB+ motoneurons in the C4 of the pig injected with FB into the thoracic part of the trapezius muscle (th-TRAP); all the neurons were VACHT+ (red; A555) and vasoactive intestinal polypeptide (VIP)- (green; A488; long arrows). Numerous VIP+ nerve fibers were distributed in the neuropile and some of them were found in a close apposition to the neurons supplying the TRAP (arrowheads); BRGDS.

Fig. 5. Confocal laser scanning microscope image showing FB+ motoneurons in the C3 of the pig injected with FB into th-TRAP; they were VACHT+ (red; A555; long arrow) but neuropeptide Y (NPY)-; green; A488; long arrow). Solitary VACHT- neurons were also encountered (short arrow). The indicated neuron (short arrow) was NPY+; BRGDS.

CHAT+ synaptic structures located on the FB+ perikarya and their neurites were also observed (Fig. 9).

A dense network of varicose VACHT-immunoreactive nerve fibers and synaptic structures were found in a close association with the VACHT+ neurons. Moreover, these VACHT+ structures surrounded VACHT+/SP+ or non-cholinergic NPY+ perikarya and their neurites (Figs. 3-8).

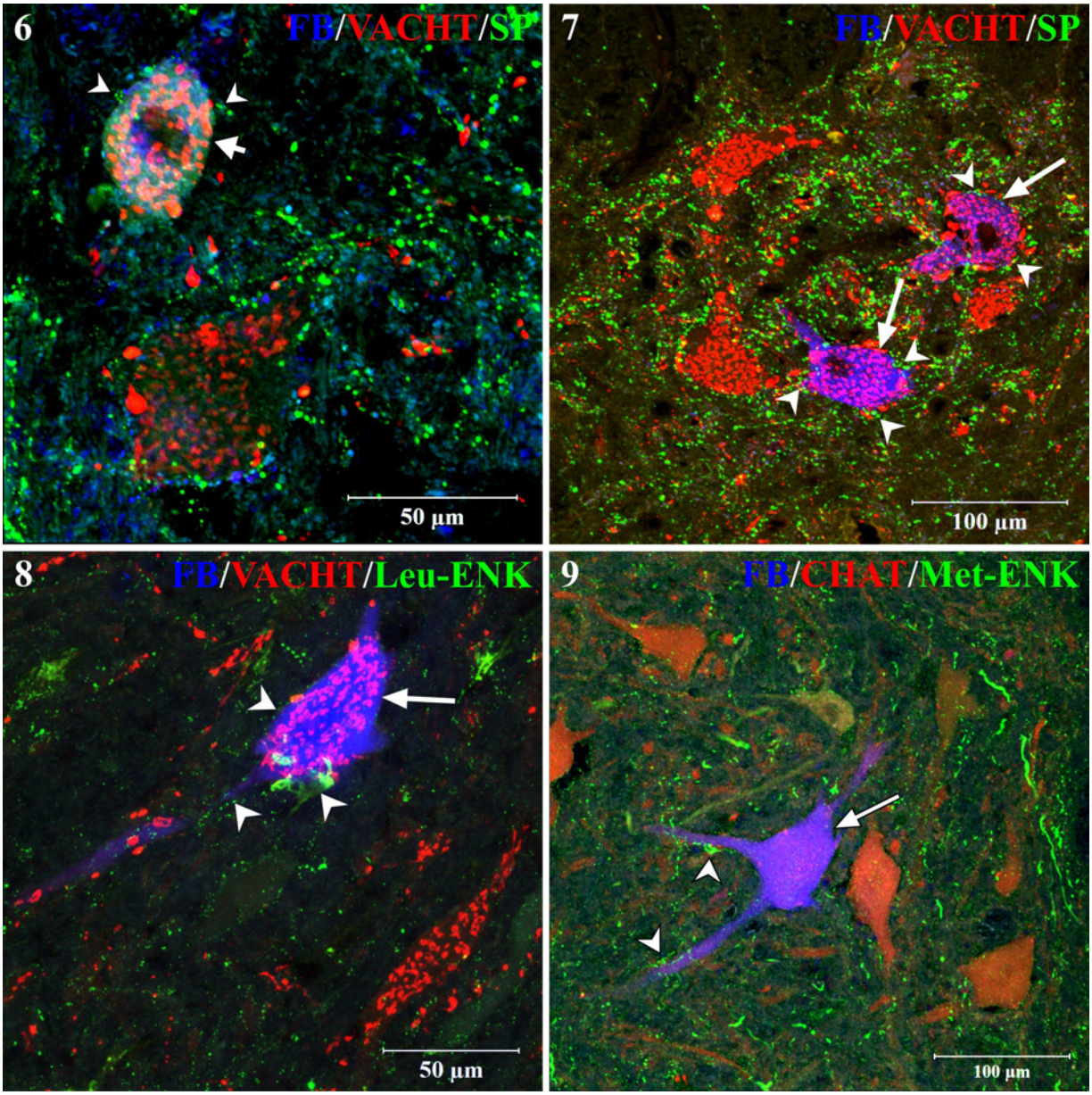

Fig. 6. Confocal laser scanning microscope image showing FB+ motoneuron in the C2 of the pig injected with FB into c-TRAP; it was simultaneously VACHT+ (red; A555) and substance P (SP)+ (green; A488; short arrow). Only very few SP+ nerve fibers were distributed in a close vicinity of FB/VACHT/SP+ neurons (arroheads); BRGDS.

Fig. 7. Confocal laser scanning microscope image showing FB+ motoneurons in the C3 of the pig injected with FB into c-TRAP; they were VACHT+ (red; A555) and SP- (green; A488; long arrows). FB+/VACHT+/SP- neurons were surrounded by numerous SP+ nerve fibers often forming basket-like structures (arrowheads); BRGDS.

Fig. 8. Confocal laser scanning microscope image showing FB+ motoneuron in the C4 of the pig injected with FB into th-TRAP, which was VACHT+(red; A555) and Leu-ENK- (green; A488; long arrow). FB+/VACHT+/Leu-ENK- neurons were closely apposed by Leu-ENK- nerve fibers (arrowheads); BRGDS.

Fig. 9. Confocal laser scanning microscope image showing FB+ motoneuron in the C3 of the pig injected with FB into th-TRAP which was simultaneously CHAT+(red; A555) but Met-ENK- (green; A488; long arrows). FB+/VACHT+/Met-ENK- neurons were surrounded by Met-ENK+ nerve fibers (arrowheads); BRGDS.

Double-labellings revealed that 68±3.60% of the cholinergic neurons expressed simultaneously CGRP (Fig. 1, 2). Moderate in number, delicate CGRP+ nerve terminals were associated with the CHAT+/CGRP+ nerve cell bodies (Fig. 2).

Small populations (5±0.58%, 3±0.58%, 1.67±0.33% and 1.33±0.33%) (Fig. 1) of FB+ neuronal somata stained for NOS (Fig. 3), VIP (Fig. 4), NPY (Fig. 5) or SP (Figs. 6, 7), respectively.

The network of SP+ nerve fibers surrounding

SP-negative perikarya was very dense (Fig. 7) while in the vicinity of SP+ neuronal somata it was sparse (Fig. 6).

VIP- or DβH-immunoreactive nerve fibers formed also very dense networks surrounding the TRAP-projecting neurons.

No PACAP-, Gal-, DβH-, Leu-5-Enk- or Met-Enk-IR (-immunoreactive) was determined in FB+ neurons. However, single Gal-, n-NOS- or NPY-expressing nerve terminals closely apposing the retrogradely labelled cholinergic neuronal somata were observed.

FB+ perikarya and visible parts of their processes were surrounded by numerous varicose Leu-5-Enk- [(Fig. 8) or Met-Enk-positive (Fig. 9)] axons. There were no differences in the immunohistochemical characteristics between motor neurons supplying both parts of the trapezius muscle.

Discussion

The present paper reports data on the chemical coding of motoneurons supplying the porcine TRAP. Immunohistochemical features of motoneurons innervating some muscles of the hind limb were described in cattle (Chiocchetti et al. 2005) and rat (Piehl et al. 1993). There are also a few papers on the chemical coding of motoneurons innervating striated muscles of the larynx (Arita et al. 1993, Hisa et al. 1998), pharynx (Eberhorn et al. 2005), muscles of the eye bulb (Eberhorn et al. 2006) and diaphragm (Holtman, Jr. et al. 1984).

In the pig, FB+ neurons supplying the c-TRAP and thoracic th-TRAP were localized within the ipsilateral ventral horn of the grey matter of the first four and five cervical neuromeres, respectively. The available literature contains only one paper dealing with the chemical coding of neurons supplying the rat TRAP (Dudek et al. 2011) and the findings reported in this paper slightly differ from those obtained in the pig. In rats, the neurons were found to be distributed in the ipsilateral nucleus ambiguous (Amb) and in the ipsilateral ventral horn of the grey matter of the spinal medulla between C1 and cranial half of C7 cervical neuromeres (Dudek et al. 2011).

The present study revealed that almost all motoneurons supplying the porcine TRAP were cholinergic in nature. CHAT- and VACHT-immunoreactivity was expressed in 99% and 97% of the retrogradelly labelled nerve cells, respectively. These findings are in congruence with data presented in papers dealing with the chemical coding of motoneurons innervating the rat TRAP (Dudek et al. 2011) reporting that nearly all of them were cholinergic (displayed CHAT-IR) and single neuronal somata revealed immunofluorescence against neuronal nitric oxide synthase (n-NOS)-IR only. The population of cholinergic motoneurons in the ventral horn of the grey matter of the spinal medulla was found in the rat (Barber et al. 1984) and mice (Houser et al. 1983). Moreover, virtually all the motoneurons located in the porcine hypoglossal nucleus expressed CHAT (Sienkiewicz et al. 2010). The lack of staining for the cholinergic markers in the solitary retrogradely labelled neurones found in the present study may be due to low levels (below the detection level) of these substances in the nerve cell bodies.

Double-labelling immunohistochemistry revealed that 68% of porcine TRAP-projecting cholinergic motoneurons displayed also IR to CGRP. The colocalization of CHAT and CGRP in motoneurons of the ventral horn of the spinal medulla (Hietanen et al. 1990), and in the nerve fibers and endplates within striated muscles was described previously in the rat (Popper and Micevych 1989, Csillik et al. 1993, Fernandez et al. 2003). These results are consistent with the observations described in this publication. A comparatively large population (60%) of motoneurons innervating TRAP was observed in the rat (Dudek et al. 2011). CGRP was expressed in half of the cholinergic motoneurons of the porcine hypoglossal nucleus (HN) (Sienkiewicz et al. 2010) and in those found in the ventral horn of the spinal cord in the pig, horse (Merighi et al. 1990) and rat (Hietanen et al. 1990). Furthermore, some muscles of the rat hind limb were also reported to be supplied by motoneurons containing immunoreactivity to CGRP (Piehl et al. 1993). Comparable populations, 73% and 79%, of CGRP+ motoneurons, were found after tracer injection into the gastrocnemius muscle and superficial digital flexor muscle, respectively, in the cattle. CGRP plays an important regulating role during the development and regeneration of neuromuscular junctions. This peptide is produced in motoneurons and transported along the axons to the endplates located on the muscle fibers equipped with CGRP receptors. It is also known that the level of CGRP increases in motoneurons innervating skeletal muscles after muscle training (Homonko and Theriault 2000). All the above-cited results of previous studies are in agreement and confirm our results.

N-NOS is generally considered to be present constitutively in most neurons and other structures including skeletal muscle fibers. It is known that NO of endogenous (Grozdanovic and Baumgarten 1999) or exogenous (Dudek et al. 2011) origin can exert several distinct effects on various aspects of skeletal muscle function, such as excitation-contraction coupling, mitochondrial energy production, glucose metabolism and autoregulation of blood flow (Grozdanovic 2001). Our

studies revealed the presence of a small population (5%) of NOS-positive retrogradelly labelled neurons innervating the porcine TRAP. In the rat, approx. half (50%) of TRAP-projecting motoneurons expressed immunoreactivity to n-NOS (Dudek and Sienkiewicz 2009). This observation indicates the existence of profound interspecies differences. Moreover, in the cat the population of neurons distributed in the location which corresponds with that of SAN was found to be NADPH- (a marker of nitrergic nerve structures)/ SP-positive (Vizzard et al. 1994). The presence of a small population of n-NOS-immunoreactive cholinergic neurons was also reported in the rat and porcine HN (Vazquez et al. 1999, Sienkiewicz et al. 2010).

VIP-, NPY- or SP-IR was present in 3%, 2% or 1%, of cholinergic motoneurons innervating the porcine TRAP, respectively. A comparatively large population of cholinergic VIP+, TRAP-projecting motoneurons was found in rats (Dudek et al. 2011). CHAT/VIP+ motoneurons were also previously described in the ventral horn of the spinal medulla in chicken embryos and in post-hatch chicks (Villar et al. 1988, Villar et al. 1989). These studies have revealed that the number of VIP+ motoneurons markedly declines at the end of the embryonic period. As mentioned above, the present study and also that performed in rats have revealed a small population of cholinergic VIP+ TRAP-projecting motoneurons. Therefore, it could be concluded that this neuronal population is a residual part of the larger cell group existing in the prenatal period. Investigations dealing with the chemical coding of motoneurons supplying rat TRAP revealed that 35% of the cholinergic neurons displayed simultaneously immunoreactivity to SP while no NPY+/cholinergic neurons were observed (Dudek et al. 2011). These differences can result from the negative influence of detergents (eg. Triton X100) on the quality of the immunohistochemical stainings. It has been revealed that they dissolve and wash out some substances from the cytoplasm (Arluison et al. 1983a, Arluison et al. 1983b). It was also mentioned in the literature that the fixation of tissues can affect the immunostaining results (Sakanaka 1992). Another possible reason could be a very fast transport of SP from the perikarya to the axons (Sakanaka 1992). Such possibility was confirmed by experiments with colchicine which allowed to visualise the presence of SP+ perikarya in the dorsal vagal nucleus (Maley and Elde 1981) and spinal cord (Senba et al. 1982). The observed interspecies differences concern not only immunohistochemical properties of neurons supplying the striated muscles. The literature in the field also indicates the presence of the significant differences in the morphology of the neuromuscular junction (Boehm et al. 2020) as well as in the morphological, physiological and biochemical characteristics of muscle fibers, even between muscles of different muscle groups in the same species (Schiaffino and Reggiani 2011).

The present study provide insights into details regarding the motor control of the clinically relevant muscle, TRAP. We have disclosed, for the first time, the chemical coding of motoneurons innervating the muscle in the pig. The results obtained differ from those gained in the rat (Dudek et al. 2011), which suggests the existence of profound interspecies differences regarding the chemical characteristics of neurons supplying mammalian striated muscles. We are convinced that the thorough knowledge of the neurotransmitters involved in the efferent innervation is a key step in understanding the potential pathomechanisms underlying common TRAP dysfunctions associated with its excessive or weakened contractility. Therefore, results of our research could help to lay the groundwork for future clinical trials and, possibly, investigations of new therapeutic agents.

References

Akamatsu FE, Ayres BR, Saleh SO, Hojaij F, Andrade M, Hsing WT, Jacomo AL **(2015)** Trigger points: an anatomical substratum. Biomed Res Int 2015: 623287.

Arita H, Sakamoto M, Hirokawa Y, Okado N **(1993)** Serotonin innervation patterns differ among the various medullary motoneuronal groups involved in upper airway control. Exp Brain Res 95: 100-110.

Arlotta M, Lovasco G, McLean L **(2011)** Selective recruitment of the lower fibers of the trapezius muscle. J Electromyogr Kinesiol 21: 403-410.

Arluison M, Conrath-Verrier M, Tauc M, Mailly P, De la Manche IS, Cesselin F, Bourgoin S, Hamon M **(1983a)** Different localizations of Met-enkephalin-like immunoreactivity in rat forebrain and spinal cord using hydrogen peroxide and Triton X-100. Light microscopic study. Brain Res Bull 11: 555-571.

Arluison M, Conrath-Verrier M, Tauc M, Mailly P, De la Manche IS, Dietl M, Cesselin F, Bourgoin S, Hamon M **(1983b)** Met-enkephalin-like immunoreactivity in rat forebrain and spinal cord using hydrogen peroxide and Triton X-100. Ultrastructural study. Brain Res Bull 11: 573-586.

Atoji Y, Kusindarta DL, Hamazaki N, Kaneko A **(2005)** Innervation of the rat trachea by bilateral cholinergic projections from the nucleus ambiguus and direct motor fibers from the cervical spinal cord: a retrograde and anterograde tracer study. Brain Res 1031: 90-100.

Barber RP, Phelps PE, Houser CR, Crawford GD, Salvaterra PM, Vaughn JE **(1984)** The morphology and distribution of neurons containing choline acetyltransferase in the adult rat spinal cord: an immunocytochemical study. J Comp Neurol 229: 329-346.

Boehm I, Alhindi A, Leite AS, Logie C, Gibbs A, Murray O, Farrukh R, Pirie R, Proudfoot C, Clutton R, Wishart TM,

Jones RA, Gillingwater TH **(2020)** Comparative anatomy of the mammalian neuromuscular junction. J Anat 237: 827-836.

Chan PK, Hems TE **(2006)** Clinical signs of accessory nerve palsy. J Trauma, 60: 1142-1144.

Chu J **(1995)** Dry needling (intramuscular stimulation) in myofascial pain related to lumbosacral radiculopathy. Eur J Phys Rehabil Med 5: 106-121.

Chiocchetti R, Grandis A, Bombardi C, Clavenzani P, Spadari A, Gentile A, Bortolami R **(2005)** Localization, morphology, and immunohistochemistry of spinal cord and dorsal root ganglion neurons that innervate the gastrocnemius and superficial digital flexor muscles in cattle. Am J Vet Res 66: 710-720.

Csillik B, Tajti L, Kovacs T, Kukla E, Rakic P, Knyihar-Csillik E **(1993)** Distribution of calcitonin gene-related peptide in vertebrate neuromuscular junctions: relationship to the acetylcholine receptor. J Histochem Cytochem 41: 1547-1555.

De Meulemeester K, Calders P, De Pauw R, Grymonpon I, Govaerts A, Cagnie B **(2017)** Morphological and physiological differences in the upper trapezius muscle in patients with work-related trapezius myalgia compared to healthy controls: A systematic review. Musculoskelet Sci Pract 29: 43-51.

Dudek A, Sienkiewicz W **(2009)** Immunohistochemical characterisation of the motoneurones supplaying trapezius muscle *(musculus trapezius)* in the rat. Abstract Book of the 29-th Congress of Polish Anatomical Society, Bydgoszcz, September 3-5, p 23.

Dudek A, Sienkiewicz W, Kaleczyc J **(2015)** Contribution of the dorsal branch of the accessory nerve to the innervation of the trapezius muscle in the pig - a retrograde tracing study. Acta Zoologica 96: 519-529.

Dudek A, Sienkiewicz W, Marczak M, Kaleczyc J **(2011)** Immunohistochemical properties of motoneurons supplying the trapezius muscle in the rat. Pol J Vet Sci 14: 199-205.

Eberhorn AC, Ardeleanu P, Buttner-Ennever JA, Horn AK **(2005)** Histochemical differences between motoneurons supplying multiply and singly innervated extraocular muscle fibers. J Comp Neurol 491: 352-366.

Eberhorn AC, Buttner-Ennever JA, Horn AK **(2006)** Identification of motoneurons supplying multiply- or singly-innervated extraocular muscle fibers in the rat. Neuroscience 137: 891-903.

Fernandez HL, Chen M, Nadelhaft I, Durr JA **(2003)** Calcitonin gene-related peptides: their binding sites and receptor accessory proteins in adult mammalian skeletal muscles. Neuroscience 119: 335-345.

Gardan D, Gondret F, Louveau I **(2006)** Lipid metabolism and secretory function of porcine intramuscular adipocytes compared with subcutaneous and perirenal adipocytes. Am J Physiol Endocrinol Metab 291: 372-380.

Grozdanovic Z **(2001)** NO message from muscle. Microsc Res Tech 55: 148-153.

Grozdanovic Z, Baumgarten HG **(1999)** Nitric oxide synthase in skeletal muscle fibers: a signaling component of the dystrophin-glycoprotein complex. Histol Histopathol 14: 243-256.

Handel SE, Stickland NC **(1986)** "Giant" muscle fibres in skeletal muscle of normal pigs. J Comp Pathol 96: 447-457.

Hietanen M, Pelto-Huikko M, Rechardt L **(1990)** Immunocytochemical study of the relations of acetylcholinesterase, enkephalin-, substance P-, choline acetyltransferase- and calcitonin gene-related peptide-immunoreactive structures in the ventral horn of rat spinal cord. Histochemistry 93: 473-477.

Hisa Y, Tadaki N, Koike S, Bamba H, Uno T **(1998)** Calcitonin gene-related peptide-like immunoreactive motoneurons innervating the canine intrinsic laryngeal muscles. Ann Otol Rhinol Laryngol 107: 1029-1032.

Hisa Y, Tadaki N, Uno T, Okamura H, Taguchi J, Ibata Y **(1994)** Calcitonin gene-related peptide-like immunoreactive motoneurons innervating the canine inferior pharyngeal constrictor muscle. Acta Otolaryngol 114: 560-564.

Holtman JR Jr, Norman WP, Skirboll L, Dretchen KL, Cuello C, Visser TJ, Hokfelt T, Gillis RA **(1984)** Evidence for 5-hydroxytryptamine, substance P, and thyrotropin-releasing hormone in neurons innervating the phrenic motor nucleus. J Neurosci 4: 1064-1071.

Homonko DA, Theriault E **(2000)** Downhill running preferentially increases CGRP in fast glycolytic muscle fibers. J Appl Physiol (1985) 89: 1928-1936.

Hou N, Du X, Wu S **(2022)** Advances in pig models of human diseases. Animal Model Exp Med 5: 141-152.

Houser CR, Crawford GD, Barber RP, Salvaterra PM, Vaughn JE **(1983)** Organization and morphological characteristics of cholinergic neurons: an immunocytochemical study with a monoclonal antibody to choline acetyltransferase. Brain Res 266: 97-119.

Iaizzo PA, Lehmann-Horn F **(1989)** The in vitro determination of susceptibility to malignant hyperthermia. Muscle Nerve 12: 184-190.

Ichikawa T, Shimizu T **(1998)** Organization of choline acetyltransferase-containing structures in the cranial nerve motor nuclei and spinal cord of the monkey. Brain Res 779: 96-103.

Jones SW, Parr T, Sensky PL, Scothern GP, Bardsley RG, Buttery PJ **(1999)** Fibre type-specific expression of p94, a skeletal muscle-specific calpain. J Muscle Res Cell Motil 20: 417-424.

Lefaucheur L **(1990)** Changes in muscle fiber populations and muscle enzyme activities in the primiparous lactating sow. Reprod Nutr Dev 30: 523-531.

Lu IC, Wang HM, Kuo YW, Shieh CF, Chiang FY, Wu CW, Tsai CJ. **(2010)** Electromyographic study of differential sensitivity to succinylcholine of the diaphragm, laryngeal and somatic muscles: a swine model. Kaohsiung J Med Sci 26: 640-646.

Lunney JK, Van Goor A, Walker KE, Hailstock T, Franklin J, Dai C **(2021)** Importance of the pig as a human biomedical model. Sci Transl Med 13 (621): eabd5758.

Maley B, Elde R **(1981)** Localization of substance P-like immunoreactivity in cell bodies of the feline dorsal vagal nucleus. Neurosci Lett 27: 187-191.

Merighi A, Kar S, Gibson SJ, Ghidella S, Gobetto A, Peirone SM, Polak JM **(1990)** The immunocytochemical distribution of seven peptides in the spinal cord and dorsal root ganglia of horse and pig. Anat Embryol (Berl) 181: 271-280.

Neumann DA **(2017)** Kinesiology of the Musculoskeletal System: Foundations for Rehabilitation. 3rd ed., Elsevier, St Louis.

Piehl F, Arvidsson U, Hokfelt T, Cullheim S **(1993)** Calcitonin

gene-related peptide-like immunoreactivity in motoneuron pools innervating different hind limb muscles in the rat. Exp Brain Res 96: 291-303.

Popper P, Micevych PE **(1989)** Localization of calcitonin gene-related peptide and its receptors in a striated muscle. Brain Res 496: 180-186.

Rock E, Kozak-Reiss G. **(1987)** Effect of halothane on the Ca2+-transport system of surface membranes isolated from normal and malignant hyperthermia pig skeletal muscle. Arch Biochem Biophys 256: 703-707.

Rock E, Sidi Mammar M, Thomas MA, Viret J, Vignon X **(1990)** Halothane-induced functional and structural modifications in sarcoplasmic reticulum membranes from pig skeletal muscle. Biochimie 72: 245-250.

Sakanaka M **(1992)** Development of neuronal elements with substance P-like immunoreactivity in the central nervous system. Ontogeny of transmitters and peptides in the CNS. Handbook of chemical neuroanatomy. Elsevier, Amsterdam-London-New york-Tokyo, pp 197-255.

Schiaffino S, Reggiani C. **(2011)** Fiber types in mammalian skeletal muscles. Physiol Rev 91: 1447-1531.

Senba E, Shiosaka S, Hara Y, Inagaki S, Sakanaka M, Takatsuki K, Kawai Y, Tohyama M **(1982)** Ontogeny of the peptidergic system in the rat spinal cord: immunohistochemical analysis. J Comp Neurol 208: 54-66.

Sienkiewicz W, Dudek A, Kaleczyc J, Chroszcz A **(2010)** Immunohistochemical characterization of neurones in the hypoglossal nucleus of the pig. Anat Histol Embryol 39: 152-159.

Swindle MM, Makin A, Herron AJ, Clubb FJ Jr, Frazier KS **(2012)** Swine as models in biomedical research and toxicology testing. Vet Pathol 49: 344-356.

Vazquez C, Anesetti G, Martinez PL **(1999)** Transient expression of nitric oxide synthase in the hypoglossal nucleus of the rat during early postnatal development. Neurosci Lett 275: 5-8.

Villar MJ, Huchet M, Hokfelt T, Changeux JP, Fahrenkrug J, Brown JC **(1988)** Existence and coexistence of calcitonin gene-related peptide, vasoactive intestinal polypeptide- and somatostatin-like immunoreactivities in spinal cord motoneurons of developing embryos and post-hatch chicks. Neurosci Lett 86: 114-118.

Villar MJ, Roa M, Huchet M, Hokfelt T, Changeux JP, Fahrenkrug J, Brown JC, Epstein M, Hersh L **(1989)** Immunoreactive calcitonin gene-related peptide, vasoactive intestinal polypeptide, and somatostatin in developing chicken spinal cord motoneurons. Eur J Neurosci 1: 269-287.

Vizzard MA, Erdman SL, Roppolo JR, Forstermann U, de Groat WC **(1994)** Differential localization of neuronal nitric oxide synthase immunoreactivity and NADPH-diaphorase activity in the cat spinal cord. Cell Tissue Res 278: 299-309.

Wessendorf MW, Elde RP (1985) Characterization of an immunofluorescence technique for the demonstration of coexisting neurotransmitters within nerve fibers and terminals. J Histochem Cytochem 33: 984-994.

Wiater JM, Bigliani LU **(1999)** Spinal accessory nerve injury. Clin Orthop Relat Res 368: 5-16.