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 retrogradely 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-inter-scapular 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 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, Lefaucheux 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-
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...
(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-enkephalin (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 antisera 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 neurones, 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,
CHAT+ synaptic structures located on the FB+ perikarya and their neurites were also observed (Fig. 9).

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

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In 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 retrogradely 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, Csilik 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 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 retrogradely 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 nitric 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.

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