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Original article

Immunohistochemical properties of motoneurons supplying the trapezius muscle in the rat

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Abstract

Combined retrograde tracing (using fluorescent tracer Fast blue) and double-labelling immunofluorescence were used to study the distribution and immunohistochemical characteristics of neurons projecting to the trapezius muscle in mature male rats (n=9). As revealed by retrograde tracing, Fast blue-positive (FB^+) neurons were located within the ambiguous nucleus and accessory nucleus of the grey matter of the spinal cord. Immunohistochemistry revealed that nearly all the neurons were cholinergic in nature [choline acetyltransferase (ChAT)-positive]. Retrogradely labelled neurons displayed also immunoreactivities to calcitonin gene-related peptide (CGRP; approximately 60% of FB^+ neurons), nitric oxide synthase (NOS; 50%), substance P (SP; 35%), Leu^5-Enkephalin (LEnk; 10%) and vasoactive intestinal polypeptide (VIP; 5%). The analysis of double-stained tissue sections revealed that all CGRP-, VIP- and LEnk-immunoreactive FB^+ perikarya were simultaneously ChAT-positive. The vast majority of the neurons expressing SP- or NOS-immunoreactivity were also cholinergic in nature; however, solitary somata were ChAT-negative. FB^+ perikarya were surrounded by numerous varicose nerve fibres (often forming basket-like structures) immunoreactive to LEnk or SP. They were also associated with some CGRP-, NOS- and neuropeptide Y-positive nerve terminals.

Key words: rat, trapezius muscle, tracing, motoneurons, immunohistochemistry

Introduction

It is well known that motoneurons supplying the trapezius muscle in the rat are located in the nucleus ambiguous (Sienkiewicz and Dudek 2010) and in the accessory nucleus of the spinal cord (Kitamura and Sakai 1982, Sienkiewicz and Dudek 2010). The localisation of motoneurons supplying the trapezius muscle in the rat is well documented but there is no information on the immunohistochemical properties of these nerve cell bodies. Immunohistochemistry of motor neurons located in the nucleus ambiguous and in the ventral horn of the spinal cord was previously described in the rat (Piehl et al. 1993, Atoji et al. 2005), and also in other species including the pig (Merighi et al. 1990, Załęcki et al. 2007, Chaillou et al. 2009) cat (Batten et al. 1989), dog (Hisa et al. 1998), horse (Merighi et al. 1990), cattle (Chiocchetti et al.

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2005) and monkey (Ichikawa and Shimizu 1998). There is a paper reporting that motoneurons located in the dorsal division of the nucleus ambiguous which innervate the striated muscle of the upper digestive tract are ChAT- and CGRP- immunoreactive (Lee et al. 1992). In the rat, pig and horse nucleus ambiguous and grey matter of the spinal cord contain a very dense network of Leu-enkephalin positive nerve fibres and terminals (Simantov et al. 1977, Merighi et al. 1990). ChAT-positive neurons distributed in the ventral horn of the gray matter of the spinal cord were described in the rat (Barber et al. 1984, Borges and Iversen 1986). Additionally, a unique population of NOS- or NADPH-positive nerve cells, localisation of which overlaps the spinal accessory nucleus was described in the cat (Vizzard et al. 1994b). There are also some contributions dealing with the immunohistochemical features of motoneurons supplying the striated muscle of the hind limb in the cattle (Chiocchetti et al. 2005) and rat (Piehl et al. 1993, Fernandez et al. 2003). Some papers describe the chemical coding of motoneurons supplying laryngeal muscles (Arita et al. 1993), oculomotor muscles (Eberhorn et al. 2005), pharyngeal muscles (Hisa et al. 1994) or diaphragm (Holtman et al. 1984), but there is no report regarding the chemical coding of motoneurons innervating the trapezius muscle in any animal species including the rat.

Materials and Methods

The study was carried out on 9 sexually mature male rats of the Wistar breed weighing approximately 250 g each. The animals were housed and treated in accordance with the rules approved by the local Ethics Commission (affiliated to the national ethics Commission for Animal Experimentation, Polish Ministry of Science and Higher Education). The tracing procedure, tissue removal, preparation and cutting, and counting of FB^+ neurones were described in detail in our previous paper (Sienkiewicz and Dudek 2010). The selected sections comprising FB^+ neurons mounted on glass slides were processed for double immunofluorescence method. The sections were washed 3x10 min. in PB, incubated 45 min. with a solution containing 10% of normal horse serum (NHS, Cappel, Warsaw, Poland) and 0,25% of Triton X-100 (Sigma, USA) dissolved in PBS and then incubated overnight in room temperature (RT) with antibodies (Table 1) diluted in PB containing 1% of normal swine serum (NSS) and 0.25% Triton X-100. After incubation with primary antiserum, the sections were washed 3x10 min. in PB and further incubated with secondary antisera for 1h in RT. After the incubation, the sections were washed 3x10 min. in PB, coverslipped with buffered glycerol and examiner under a fluorescent microscope. The distribution of immunoreactivities in the tissues studied was verified by comparison of currently observed structures with corresponding areas described in the atlas of the rat brain (Paxinos and Watson 1998). Control of specificity of staining was performed by preabsorbtion of a diluted antiserum with 20 μg/ml of an appropriate antigen, which abolished the specific immunoreaction completely. In addition, experiments were carried out in which the primary antiserum was replaced by non-immune serum, or by PBS, in order to verify the specificity of particular immunoreactions.

Results

As previously described (Sienkiewicz and Dudek 2010) tracer-containing neuronal somata were localised in the ipsilateral nucleus ambiguous. The neurons were multipolar and with relatively large diameter ranging from 40 to 80 μm.

FB^+ perikarya were also found in the spinal medulla. They were distributed between the C_1 and cranial half of C_7 cervical neuromers. FB^+ neurons were located in the ipsilateral area ventrolaterally to the central canal (IX-th Rexed lamina). This localisation overlaps the localisation of the spinal accessory nerve nucleus. All FB^+ neurons were found in the ipsilateral nucleus. The neurons were multipolar with relatively large diameter ranging from 60 to 80 μm. The majority of the perikarya were of multipolar or triangular shape. Nearly all (above 95%) FB^+ neurons were cholinergic in nature (ChAT-positive) (Figs. 1-6 a-b). Double-stainings revealed that approx. 60% of all cholinergic neurons supplying the trapezius muscle found within the Amb (Figs. 1a,b,c) and spinal accessory nucleus (SAN) (Figs. 2a,b,c) displayed simultaneously immunoreactivity to CGRP. Small numbers of nerve fibres immunoreactive to CGRP were observed within the nuclei. SP-immunoreactivity was determined in approx. 35% FB^+ nerve cell bodies in both nuclei – Amb (Figs. 3a,b,c) and SAN, and also in numerous varicose nerve fibres forming dense networks surrounding ChAT-positive nerve cell bodies. The vast majority of SP-positive neurones were also cholinergic in nature. However solitary somata were CHAT-negative (Figs. 3a,b,c). Leu-5-Enk-immunorecativity was observed within numerous varicose nerve fibres forming "basket-like structures" around $FB^+/CHAT^+$ perikarya (Figs. 4a,b,c). Some neurons (approx. 10%) supplying the trapezius muscle localised in Amb (Figs. 4a,b,c) and SAN contained immunoreactivity to Leu-5-Enk. All of them were simultaneously CHAT-positive. About 50% of FB^+ neurons found in Amb (Figs. 5 a,b,c) and SAN (Figs. 6 a,b,c) contained immunoreactivity to NOS. Nearly all NOS-immunoreactive neurons were simultaneous-

Table 1. Antisera used in the study.

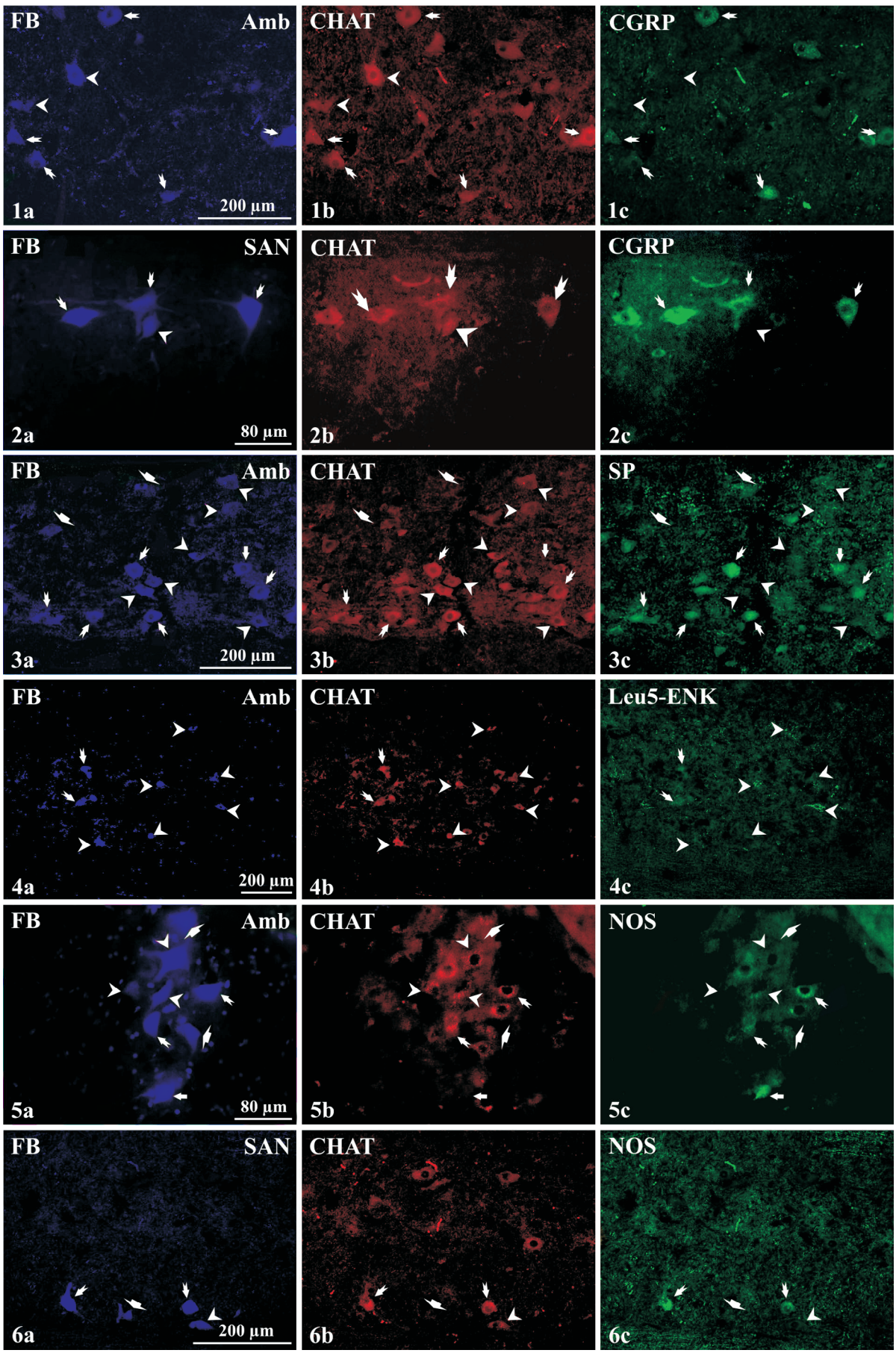
Antigen	Host	Type	Dilution	Cat. No.	Lot/Batch	Supplier
Primary antisera						
ChAT	goat	polyclonal	1:50	AB144P	LV1480420	Milipore
CGRP	rabbit	polyclonal	1:2000	11535	2659F	Cappel
SP	rabbit	polyclonal	1:600	LSC42165	14326	Lifespan
NOS	rabbit	polyclonal	1:2000	11736	8648C	Cappel
Leu-5-Enk	rabbit	polyclonal	1:500	RPN1552	5	Amersham
NPY	rabbit	polyclonal	1:400	NA1233	Z07336	Biomol
VIP	rabbit	polyclonal	1:2000	ab22736	552916	abcam
Secondary antisera						
Host		Fluorochrom	Dilution	Code	Lot	Supplier
Donkey-anti-goat IgG (H+L)		Alexa Flour 546	1:500	A11056	399681	Invitrigen
Donkey-anti-rabbit IgG (H+L)		Alexa Fluor 488	1:500	A21206	57542A	Invitrogen

ly CHAT-positive, but single FB^+ neurones were NOS-positive only (Figs. 5a,b,c). Nerve fibres immunoreactive to NOS were moderate in number, delicate, often running in a close vicinity of the nerve cell bodies (Figs. 6 a,b,c). Immunoreactivity to NPY was found in a small number of nerve fibres; some of them were closely apposed to ChAT-positive perikarya. Single (less than 5%) $FB^+/CHAT^+$ neurones contained also immunoreactivity to VIP

Discussion

In the present paper, immunohistochemical properties of motoneurons supplying the trapezius muscle in the rat were described. The available literature contains limited number of contributions dealing with the chemical coding of neurones supplying striated muscles of the trunk and limbs, but the information on the neurons innervating striated muscle of the head is slightly more comprehensive.

All textbooks in the field define vertebrate motoneurons as cholinergic. There are some papers describing cholinergic population of neurons and fibres within motor nuclei of cranial nerves, and in the ventral horn of the grey matter of the spinal cord in the rat (Houser et al. 1983, Barber et al. 1984, Borges and Iversen 1986). Calcitonine gene-related peptide (CGRP) is known to co-localize in cholinergic neurons of both cranial and spinal motor nuclei. This peptide participates in the process of synaptic transmission at the neuromuscular junction of cranial motor neurons (Moore 1989). In a previous study, co-localization of CHAT and CGRP in vagal motoneurons, located in the nucleus ambiguous supplying striated muscle of the upper digestive tract (Lee et al. 1992) was determined. Similar observations regarded neurons innervating striated laryngeal and pharyngeal muscles (McWilliam et al. 1989). Neurons supplying some hind limb muscles in the rat located in IX-th Rexed's lamina are CGRP-immunoreactive (Piehl et al. 1993). This observation has been confirmed by similar studies performed on cattle (Chiocchetti et al. 2005). In our study, approximately 60% of labelled CHAT positive motoneurons were also immunoreactive for CGRP. These results support the assumption on the role of CGRP as a transmitter or modulator in efferents innervating striated muscles. Substance P-positive fibres and buttons were found around the laryngeal motoneurons within the nucleus ambiguous in the rat (Sun et al. 2003) and cat (Holtman, Jr. 1988). These authors have suggested that substance P has only a low significance in the control of laryngeal motoneurons. Motoneurons supplying the gastrocnemius and superficial digital flexor muscles in the cow are SP-negative but they are surrounded by SP-positive nerve fibres. Surprisingly, in our study besides the dense network of SP-positive fibres surrounding CHAT-positive motoneurons supplying the trapezius muscle, we also found that approx. 35% of these nerve cell bodies in both nuclei studied (Amb and SAN) contain immunoreactivity to SP. The vast majority of them contained CHAT, but also single FB/SP-positive only neurons have been found. Until now, reports confirming the presence of SP-immunoreactive cells within the ventral horn of the spinal cord are very scarce (Senba et al. 1982), but there are several papers describing the presence of this peptide in brainstem nuclei including the dorsal motor nucleus of the vagus, and hypoglossal nucleus (Maley and Elde 1981, Del et al. 1983, Del et al. 1984, Block et al. 1987). The main reason of the lack of immunoreactivity to SP within motoneurons reported in previous papers is probably a very fast transport of the peptide from perikarya to the axons (Sakanaka 1992). Such possibility was confirmed by experiments with colchicines


FB
Amb
1a
200 µm
CHAT
1b
CGRP
1c
FB
SAN
2a
80 µm
CHAT
2b
CGRP
2c
FB
Amb
3a
200 µm
CHAT
3b
SP
3c
FB
Amb
4a
200 µm
CHAT
4b
Leu5-ENK
4c
FB
Amb
5a
80 µm
CHAT
5b
NOS
5c
FB
SAN
6a
200 µm
CHAT
6b
NOS
6c

which allowed to visualise numerous SP-positive neuronal somata within the dorsal vagal nucleus (Maley and Elde 1981) and spinal cord (Senba et al. 1982). Another explanation mentioned in the literature is fixation of the tissue, which can influence the results of the stainings (Sakanaka 1992). The present study has revealed a dense network of varicose nerve fibres immunoreactive to Leu-5-Enk within both SAN and Amb nuclei. $CHAT^+$ perikarya supplying the trapezius were surrounded by enkephalinergic "basket-like structures". Similar findings (dense network of opioidergic fibres around motoneurons) have been described in papers dealing with immunohistochemical characteristics of neuronal structures in the ventral horn of the spinal cord in the pig and horse (Merighi et al. 1990), rat (Sar et al. 1978, Finley et al. 1981), and birds (Atsumi and Sakamoto 1987, Du and Dubois 1988). We have found some, approx. 10% of Leu-5-Enk positive neurons in both nuclei supplying the trapezius muscle and all of them were CHAT-immunoreactive. In the available literature there are only few papers reporting the presence of opioids in motoneurons (Sar et al. 1978, de Lanerolle et al. 1981). Difficulties with visualisation of the opioid-containing nerve cells can be due to the rapid transport of the peptide from the perikarya to the axons, which has been verified by experiments using colchicine (Sar et al. 1978, Uda et al. 1985). This substance blocks transport of neuropeptides from the perikarya to the nerve endings and thus increases intensivity of the staining. Application of detergents such as Triton X100 during immunostainings also lowers the quality of staining due to dissolving and rinsing of non-protected peptide out of the cell cytoplasm (Arluison et al. 1983a, Arluison et al. 1983b). About 50% of $FB^+/CHAT^+$ neurons in both nuclei were NOS-immunoreactive. Previously a unique group of NADPH-d-positive and NOS-immunoreactive motoneurons in ventral horns of cervical neuromers of the spinal cord in the cat has been described. The localisation of these neurons corresponds with the localisation of SAN (Vizzard et al. 1994a). Moreover, we have observed a moderate number of delicate NOS-positive fibres running very closely to motoneurons, and a small number of NPY-positive fibres found in a very close apposition to CHAT-positive neurons. These observations confirm results of previous studies dealing with immunohistochemistry of IX-th Rexed's lamina in the horse and pig (Merighi et al. 1990). We have also found a few (approx. 5% of all FB-positive) CHAT/VIP-immunoreactive motoneurons. CHAT/VIP-positive motoneurons were described previously in the ventral horn of the spinal cord of chicken embryos and in post-hatch chicks (Villar et al. 1988, Villar et al. 1989). These studies have revealed that the number of VIP-positive motoneurons markedly declines at the end of the embryonic period. As mentioned above, we have found only few VIP-positive cells, so, it can be concluded that these neuronal somata are a residual part of larger cell group existing in the prenatal period.

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Fig. 1. FB^+ perikarya in nucleus ambiguous (Amb) (Fig. 1a). All the cells contained immunoreactivity to CHAT (Fig. 1b) and the majority of them were simultaneously immunoreactive to CGRP (Fig. 1b,c, dovetail arrows), but also some only $FB^+/CHAT+$ cells are visible (arrowheads).

Fig. 2. FB-positive neurons within spinal accessory nucleus (SAN) (2a). Most of FB^+ neurons displayed immunoreactivity to CHAT and CGRP (Figs. 2b,c, dovetail arrow) and one neuron expressed immunoreactivity to CHAT only (Figs. 2b,c, arrowhead).

Fig. 3. FB^+ neurons within Amb (3a) stained for CHAT and SP. Wide arrows show FB^+ only cells (3a). A large number of the stained neurons displayed immunoreactivity simultaneously to CHAT and SP (Figs. 3b,c, dovetail arrows). Note the presence of FB^+ neurons immunoreactive to CHAT only (arrowheads) and a single neuron was SP-positive (Figs. 3b,c, arrow).

Fig. 4. FB^+ neurons within Amb (4a) stained for CHAT (4b) and Leu-5-Enk (4c). Some FB^+ neurons were simultaneously CHAT and Leu-5-Enk-positive (Figs. 4b,c, dovetail arrows) whereas the majority of FB^+ cells were immunoreactive to CHAT (arrowheads). Note the presence of "basket-like structures" formed by Leu-5-Enk-positive nerve fibres around $FB^+/CHAT^+$ perikarya (Fig. 4c).

Fig 5. FB^+ neurons within Amb (5a) stained for CHAT (5b) and NOS (5c). Single neurons were FB^+ only (wide arrows). Approximately 50% of FB^+ neurons contained immunoreactivity to CHAT and NOS (Figs.5b,c, dovetail arrows). Some neurons were CHAT-positive only (arrowheads). A single FB^+ neuron displayed immunoreactivity to NOS only (Figs. 5d,c, arrow).

Fig. 6. FB^+ neurons within SAN (6a) stained for CHAT (6b) and NOS (6c). FB^+ neurons expressed immunoreactivity to CHAT and NOS (Figs. 6b,c, dovetail arrows), the remaining neuronal somata were CHAT-positive only (arrowheads).

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