

A comparison of the distribution and morphology of ChAT-, VACHT-immunoreactive and AChE-positive neurons in the thoracolumbar and sacral spinal cord of the pig

J. CALKA¹, M. ZALECKI¹, K. WASOWICZ¹, M.B. ARCISZEWSKI², M. LAKOMY¹

¹Faculty of Veterinary Medicine, University of Warmia and Mazury, Olsztyn, Poland

²Faculty of Veterinary Medicine, University of Life Sciences, Lublin, Poland

ABSTRACT: Present knowledge concerning the organization of cholinergic structures of the spinal cord has been derived primarily from studies on small laboratory animals, while there is a complete lack of information concerning its structure in the pig. In the present study we employed choline acetyltransferase (ChAT) and vesicular acetylcholine transporter (VACHT) immunocytochemistry and acetylcholinesterase (AChE) histochemistry to identify the cholinergic neuronal population in the thoracolumbar and sacral spinal cord of the pig. The distribution of ChAT-, VACHT- and AChE-positive cells was found to be similar. Distinct groups of cholinergic neurons were observed in the gray matter of the ventral horn, intermediolateral nucleus, intermediomedial nucleus as well as individual stained cells were found in the area around the central canal and in the base of the dorsal horn. Double staining confirmed complete colocalization of ChAT with AChE in the ventral horn and intermediolateral nucleus although in the intermediomedial nucleus only 64% of the AChE-positive neurons expressed ChAT-immunoreactivity, indicating unique, region restricted, diversity of ChAT and AChE staining. Our results revealed details concerning spatial distribution and morphological features of the cholinergic neurons in the thoracolumbar and sacral spinal cord of the pig. We also found that the pattern of distribution of cholinergic neurons in the porcine spinal cord shows great similarity to the organization of the cholinergic system in other mammalian species studied.

Keywords: porcine; cholinergic system; choline acetyltransferase; vesicular acetylcholine transporter; acetylcholinesterase; central nervous system

Distribution of acetylcholinesterase (AChE), the degradative enzyme for acetylcholine, has primarily enabled to describe the cholinergic neuronal network in the spinal cord of a number of species (Galabov, 1976; Galabov and Davidoff, 1976; Malatova, 1983; Satoh and Fibiger, 1985). Later development of immunocytochemistry for choline acetyltransferase (ChAT), the enzyme catalyzing the formation of acetylcholine, provided a commonly accepted specific marker of cholinergic structures within the nervous tissue (Fonnum, 1973). Recently, vesicular acetylcholine transporter (VACHT), an additional specific marker for cholinergic neurons, allowed the immunocytochemical identification of

acetylcholine-containing neurons in the nervous system (Arvidsson et al., 1997).

An immunocytochemical analysis revealed cytoarchitectonics of the cholinergic system in spinal cords of several species: rat (Borges and Iversen, 1986; Ichikawa et al., 1997), mouse (Chan-Palay et al., 1982), guinea pig (Davidoff et al., 1989), rabbit (Kan et al., 1978), cat (Kimura et al., 1981) and humans (Muroishi et al., 2000; Oda and Nakanishi, 2000). Renewed interest in the structure and function of the spinal cholinergic neuronal system has been increased in recent years by the study associated with impairment of cholinergic functions. Indeed, a reduced activity of cholinergic motoneurons (Oda,

1999) and autonomic neurons (Baltadzhieva et al., 2005) was found in amyotrophic lateral sclerosis, a neurodegenerative disease that results in progressive degeneration of motor neurons of the brain and spinal cord. Although central cholinergic neurons have already been studied in the porcine hypothalamus (Calka et al., 1993), the morphological details of cholinergic structures within the porcine spinal cord have not been systematically investigated until now. In fact, the pig due to its embryological, anatomical and physiological similarities to the humans constitutes an especially valuable species for bio-medical research (Swindle et al., 1992). At present, however, the precise anatomical characteristic of cholinergic neurons in the porcine spinal cord remains unknown, precluding the utilization of the pig as an animal model for neurodegenerative studies.

We have therefore applied comparatively the choline acetyltransferase and vesicular acetylcholine transporter immunocytochemistry and acetylcholinesterase histochemistry to identify the distribution and morphology of cholinergic neurons and fibres within the porcine thoracolumbar and sacral spinal cord, the region where both motor and autonomic nuclei are localized. In addition, the coexpression of ChAT-immunoreactivity in AChE-positive neurons was investigated.

MATERIAL AND METHODS

All experimental procedures were in agreement with the Polish "Principles of Laboratory Animal Care" (NIH publication No. 86-23, rev. 1985) and the specific national laws on experimental animal handling.

Three sexually immature gilts of the Large White Polish race (body weight ca. 20 kg) obtained from a commercial fattening farm (Szczesne, Poland) were used for the study. All the animals were deeply anaesthetized with pentobarbital (Vetbutal, Biowet, Poland, 30 mg/kg of body weight) and perfused transcardially with a 4% solution of paraformaldehyde in 0.1M phosphate buffer (PB; pH 7.4). After perfusion the spinal cords were collected from all the animals studied, postfixed in the same fixative as used for perfusion (2 h), rinsed in PB overnight and finally transferred to and stored in an 18% buffered (pH 7.4) sucrose solution until further processing. Transverse frozen sections of selected spinal segments (Th1, Th7, Th13, L2, L5, S1) were

then cut in a cryostat to a thickness of 20 μ m. Serial sections were mounted on chrome alum-gelatine-coated slides and air-dried.

Neighbouring sections were subjected to single immunostaining for choline acetyltransferase (ChAT) or vesicular acetylcholine transporter (VACHT), respectively. The slides were air-dried, hydrated in phosphate-buffered saline (PBS) and blocked with a mixture containing 0.25% Triton X-100, 1% bovine serum albumin, and 10% normal goat serum in PBS for 1 h at room temperature. After rinsing in PBS (3×10 min) selected sections were incubated with a rabbit polyclonal anti-ChAT antibody (Chemicon International Inc.; Bruce et al., 1985; German et al., 1985) diluted to 1 : 5 000. Neighbouring sections were incubated with a rabbit polyclonal anti-VACHT antibody (Phoenix Pharmaceuticals Inc.; Arvidsson et al., 1997; Miampamba et al., 2001) at dilution 1 : 4 000. Incubation was carried out overnight at room temperature and the following morning the slides were rinsed in PBS (3×10 min). Biotinylated secondary antisera (Vector Laboratories Inc.) directed against the host of primary antisera at dilution 1 : 400 were then incubated for 1 h at room temperature. After rinsing in PBS (3×10 min) the sections were incubated with CY3-conjugated streptavidin (Jackson ImmunoResearch Laboratories Inc.) at dilution 1 : 4 000 for 1 h at room temperature. Finally, the slides were rinsed in PBS (3×10 min) and then coverslipped with carbonate-buffered glycerol (pH 8.6).

The slides were then analyzed under a fluorescent microscope (Axiophot, Zeiss, Germany), photographed with a confocal microscope (BIO-RAD) and the co-ordinates of the microscope stage were recorded. Selected slides containing ChAT-positive neurons were then rinsed in PBS overnight, and, after removing the cover glass, incubated for detection of AChE activity according to the method of Karnovsky and Roots (1964). During the incubation tetraisopropylpyrophosphoramidate (TIPA), an inhibitor of non-specific cholinesterases, was present in the incubation medium at a concentration of 10^{-6} M. On completion of the staining, the sections were rinsed in PBS (3×10 min) and coverslipped with Entellan. The slides were then analyzed under the microscope (Axiophot, Zeiss, Germany). The recorded co-ordinates and photographs taken previously of immunofluorescent positive neurons were used to establish the same sites in the sections. The corresponding locations were photographed and

the pictures were matched. For a statistical analysis all single labelled AChE-positive neurons as well as double labelled AChE/ChAT-stained cells were counted on ten representative slides originating from the intermediomedial nucleus (S1) of each experimental animal.

The omission of primary antisera as well as their replacement with normal rabbit serum were applied as specificity control. The specificity of AChE histochemistry was confirmed by the incubation

of the sections in a medium devoid of the reaction substrate acetylthiocholine iodide.

RESULTS

ChAT-immunoreactivity

Neurons showing ChAT immunoreactivity were disclosed at all segmental levels through-

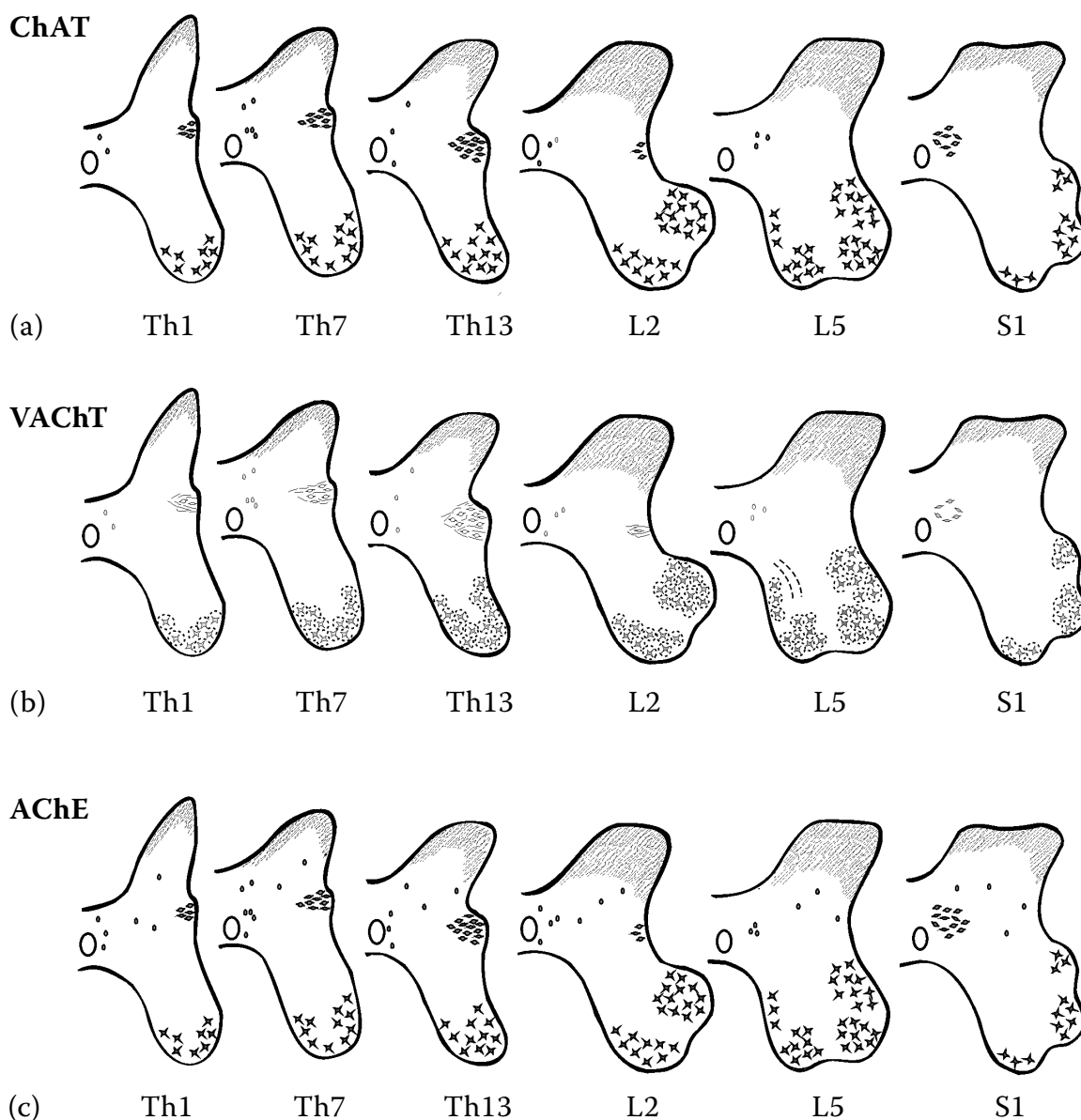


Figure 1. Distribution of positively labelled nerve cells and fibres identified by ChAT-immunocytochemistry (a), VAcHT-immunohistochemistry (b) and AChE-histochemistry (c) in selected spinal segments (Th1, Th7, Th13, L2, L5, S1) of the thoracolumbar and sacral spinal cord of the pig. The drawings were prepared by averaging the counts from three pigs

out the studied regions of the porcine spinal cord (Figure 1a). These neurons were encountered in the gray matter, while the white matter was devoid of stained cells. The most numerous concentration of the ChAT-immunoreactive perikarya was observed in the ventral horn where, depending on the segmental level, the neurons were either evenly scattered (see Th1, Th7, Th13) or concentrated into

small groups composed of three up to several cells (see L2, L5, S1). The highest number of stained somata was localized in the ventral horn at the L5 level where the cells constituted four distinguished groups occupying the mediolateral aspect of the horn.

In the studied segments neurons of the ventral horn were deeply ChAT-immunoreactive

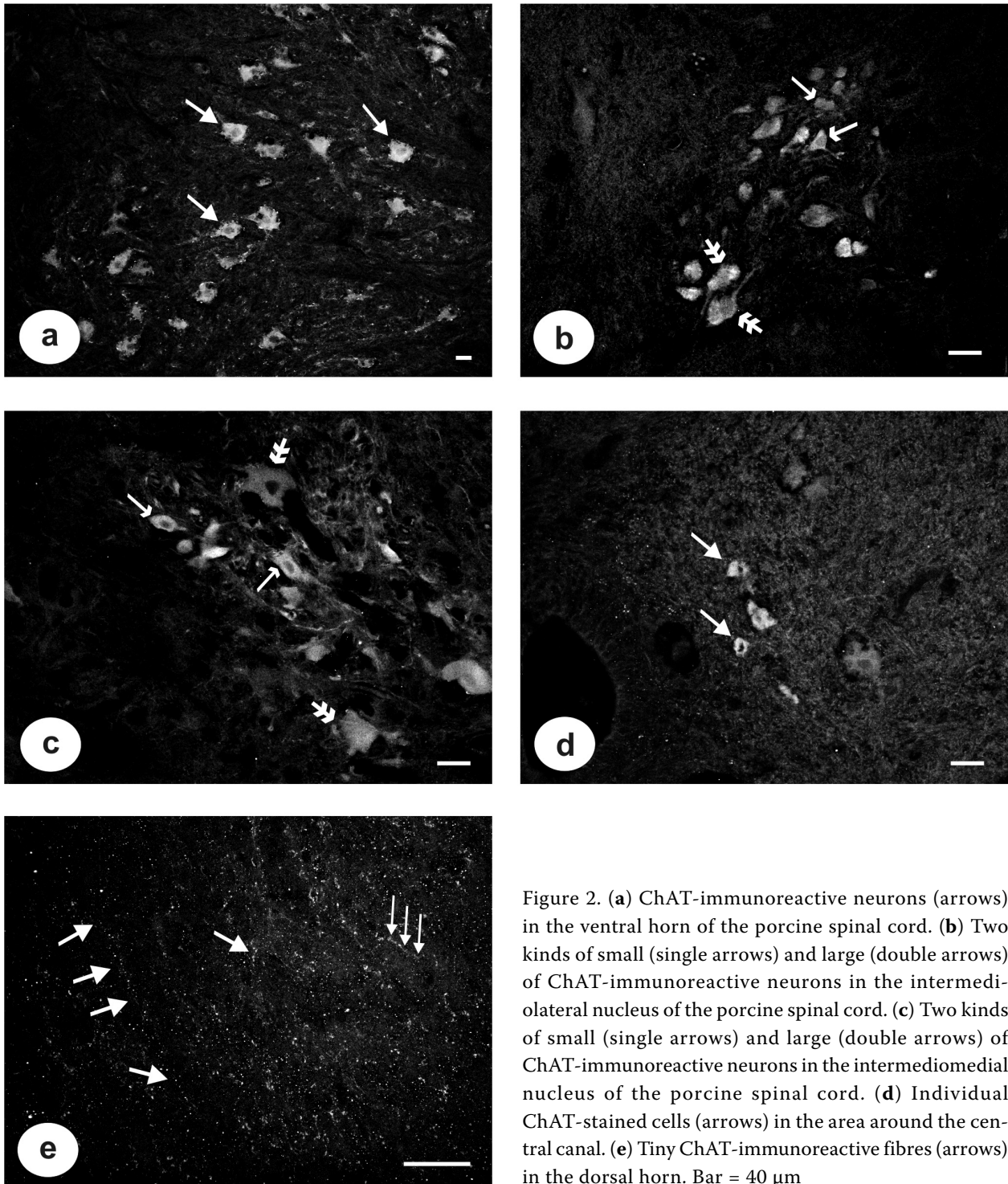


Figure 2. (a) ChAT-immunoreactive neurons (arrows) in the ventral horn of the porcine spinal cord. (b) Two kinds of small (single arrows) and large (double arrows) of ChAT-immunoreactive neurons in the intermediolateral nucleus of the porcine spinal cord. (c) Two kinds of small (single arrows) and large (double arrows) of ChAT-immunoreactive neurons in the intermediomedial nucleus of the porcine spinal cord. (d) Individual ChAT-stained cells (arrows) in the area around the central canal. (e) Tiny ChAT-immunoreactive fibres (arrows) in the dorsal horn. Bar = 40 μ m

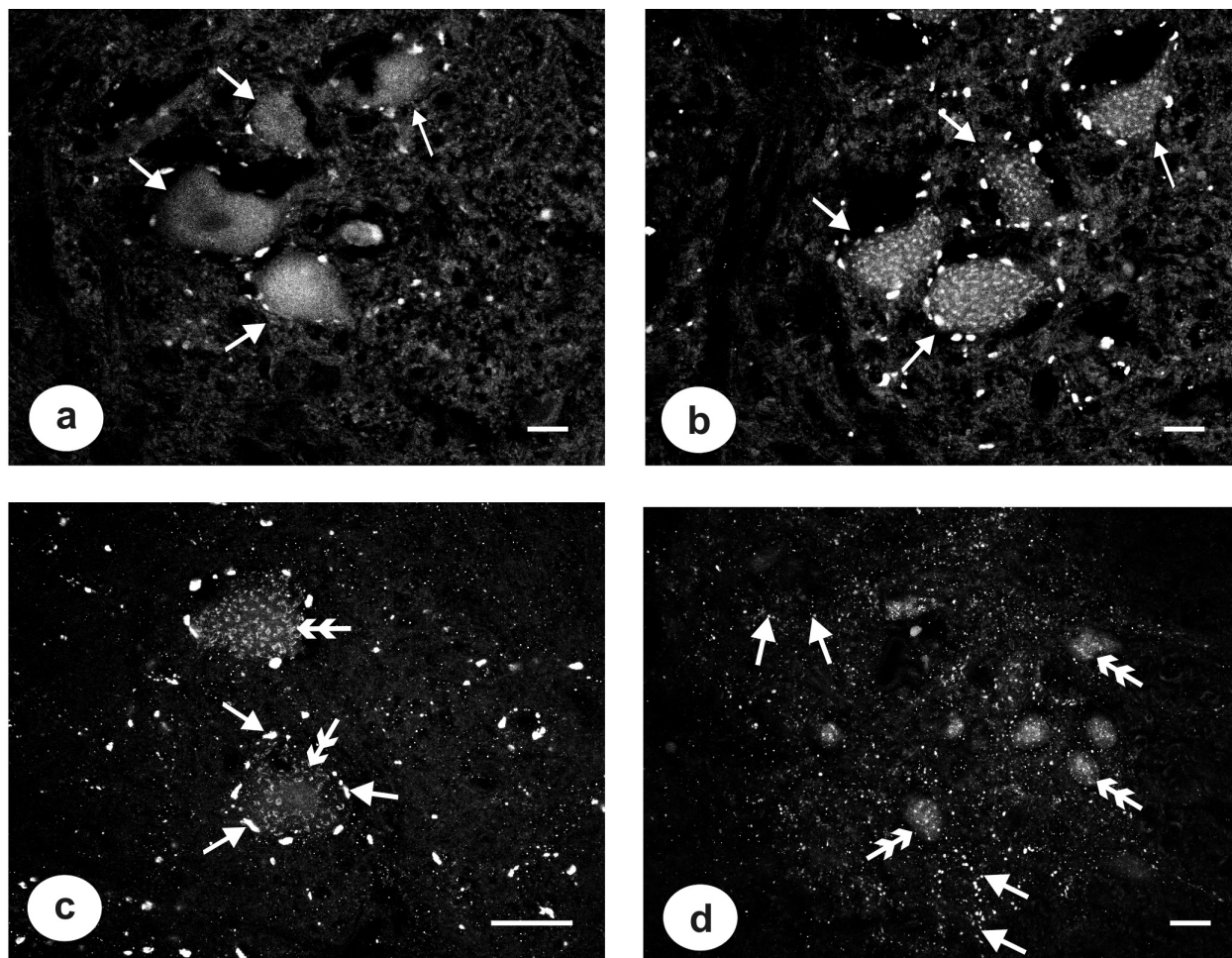


Figure 3. (a) (b) A pair of photomicrographs demonstrating different nature of the ChAT and VACHT stainings of the same ventral horn cells on the consecutive sections; (a) “smooth” ChAT-positive motor neurons (arrows) and (b) “clumpy” VACHT-immunoreactive cells (arrows). (c) Numerous large bouton-like structures, intensely stained for VACHT (single arrows), surround in close proximity the weaker-stained motoneuronal ventral horn perikarya (double arrows). (d) The VACHT immunoreactive perikarya (double arrows) and varicose fibres (single

(Figure 2a). They were mostly multipolar neurocytes with large cell bodies 40 to 80 μm in diameter. A broad rim of cytoplasm surrounded large oval nuclei. The majority of the motor neurons were accompanied by thin varicose ChAT-immunoreactive processes surrounding their perikarya.

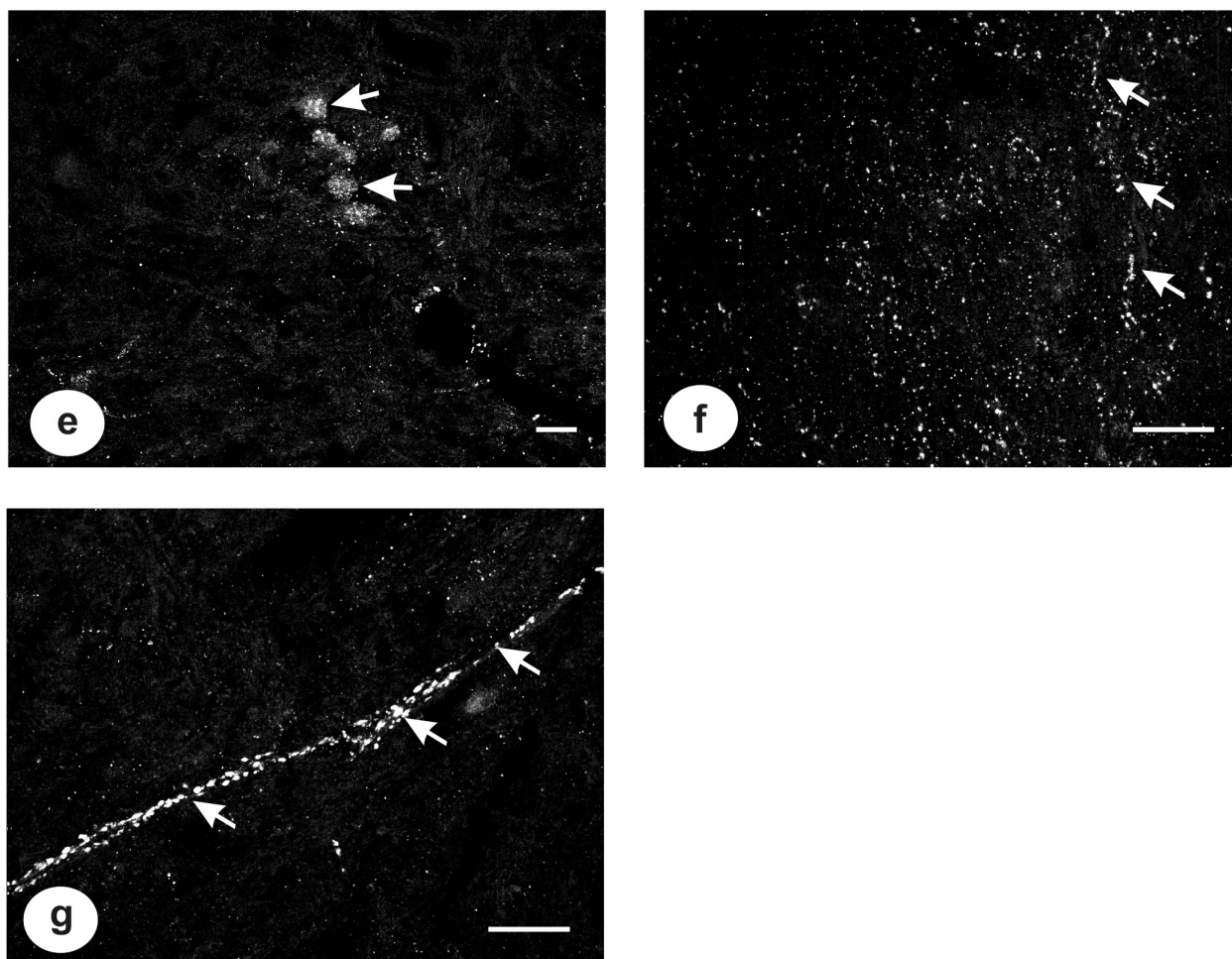
From level Th1 to level L2 of the spinal cord the intermediolateral column of gray matter contained a concentration of ChAT-immunoreactive neurons constituting an intermediolateral nucleus (Figure 2b). The neurons were unevenly distributed throughout the column, reaching a maximum number of approximately sixteen cells at the Th13 level of the cord. The ChAT-positive cells of the intermediolateral nucleus fell into two categories: large, moderately labelled cells resembling those of the ventral horn 30 to 40 μm in diameter, and

smaller, more numerous intermingled neurons of oval, triangular or spindle morphology. The smaller cells displayed oval nuclei surrounded by a narrow band of intensely stained cytoplasm.

In the sacral segments (see S1) a noticeable concentration of ChAT-immunoreactive perikarya located laterally to the central canal, forming an intermediomedial nucleus was observed (Figure 2c). The morphology and size of the neurons in this nucleus resembled those of the intermediolateral nucleus although the population of large neurons reached 60 μm in diameter.

Moreover, individual stained cells were found in lamina X in the area around the central canal as well as at the base of the dorsal horn (Figure 2d).

The ChAT-immunoreactive nerve fibres, in addition to those accompanying motor neurons in



Continuing to Figure 3

arrows) in the region of the intermediolateral nucleus. (e) Weak VAcHT-immunoreactivity in neurons (arrows) of the intermediomedial nucleus. (f) VAcHT-immunoreactive fibres (arrows) in the dorsal horn of the spinal cord. (g) Prominent VAcHT-immunoreactive nerve fibres (arrows) crossing the centro-medial part of the ventral horn at the L5 level. Bar = 40 μ m

the ventral horn, were observed throughout the intermediolateral column. Tiny immunoreactive fibres appeared also in the dorsal horn (Figure 2e), exhibiting a different concentration and location depending on the cross-section level. They were

present in laminae I and II at the level of Th1, Th7 and Th13, occupied laminae I, II and III at L2 and laminae I, II, III and IV at lumbar level L5. In the sacral segment S1 the fibres were found in the dorsal aspect of the dorsal horn.

Table 1. Percentage of double labeled AChE/ChAT-positive neurons in comparison with the AChE counts (mean \pm S.E.M.)

Nucleus	AChE	AChE/ChAT	Coexistence (%)*
Intermediomedialis	123 \pm 2.89	78.33 \pm 0.88	63.7 \pm 0.81

AChE = mean number of AChE-positive neurons in the intermediomedial nucleus of the porcine spinal cord counted on ten slides per animal

AChE/ChAT = mean number of AChE-positive cells expressing ChAT-immunoreactivity counted on the same slides

*% indicates the percentage of double labelled neurons showing coexistence when compared with the AChE counts

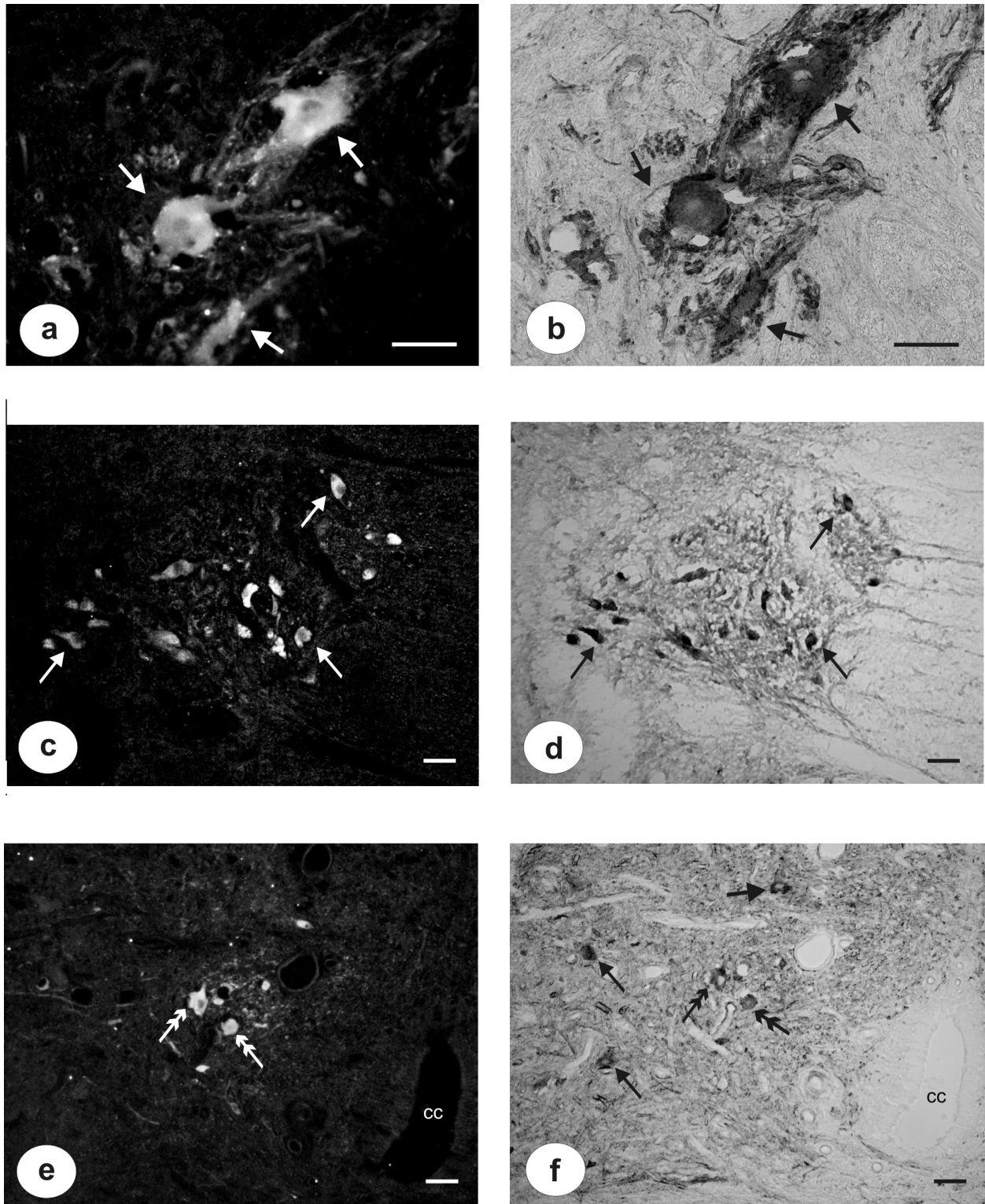


Figure 4. (a) (b) Photomicrographs demonstrating the complete colocalization of ChAT-immunoreactivity (a) and AChE-enzymatic activity (b) in the same ventral horn motor neurons (arrows). (c) (d) Full coexistence of ChAT (c) and AChE (d) in the perikarya (arrows) of the intermediolateral nucleus. (e) (f) Incomplete colocalization of the ChAT- (e) and AChE-activity (f) in the intermediomedial nucleus. All ChAT-immunoreactive perikarya were found to be AChE-positive (double arrows), while some AChE-stained neurons appeared to be ChAT-negative (single arrows); cc = central canal. Bar = 40 μ m

VACHT-immunoreactivity

In general, the pattern of the distribution of perikarya and fibres containing VACHT in the studied regions of the spinal cord was similar to that described above for ChAT (Figure 1b). Nevertheless, the cellular pattern of VACHT-immunostaining differed from that of ChAT-immunolabelling. While the ChAT-immunoreactive elements were filled with homogeneous immunoprecipitate, the VACHT-immunoreactive material appeared as “clumps” distributed within the cytoplasm (Figure 3 a,b).

The immunostaining of the ventral horn motor neurons for VACHT showed a similar characteristic of spatial distribution as ChAT staining, although the intensity of staining of the perikarya by the anti-VACHT antibody was generally weaker than that for anti-ChAT antiserum. In addition, the VACHT immunostaining of motor neurons showed a gradual increase in the staining intensity, depending on the specific rostro-caudal location of the neurons. The weakest staining of Th1 segment increased slightly through Th7 and Th13, reaching a maximum at segments L2 and L5. Numerous large bouton-like structures, intensely stained for VACHT, surrounded in close proximity the weaker-stained motoneuronal perikarya (Figure 3c). Some of the VACHT-immunoreactive boutons constituting perineuronal basket-like structures appeared likely to establish direct contacts with the perikarya.

VACHT immunoreactive perikarya and varicosities were present in the region of the intermediolateral cell column (Figure 3d) of spinal segments Th1-L2. Although the distribution and morphology of immunolabelled structures was similar to ChAT-immunoreactive elements previously labelled in this location, the intensity of VACHT-immunoreactivity in labelled somata appeared very weak. The VACHT-immunoreactive perikarya of the nucleus were found to be weaker-stained throughout the entire extent of the nucleus. Intensely stained, delicate, varicose processes intermingled between the cells. Some of the fibres were in close contact with the neurons.

In spinal segment S1, VACHT-immunoreactivity was present in a distinct population of neurons forming an intermediomedial nucleus (Figure 3e). The stained somata were surrounded by weakly labelled and scarce bouton-like processes.

The light microscopic analysis revealed numerous, VACHT-immunoreactive varicose fibres distributed throughout the prevailing area of the gray matter of

the spinal cord. These fibres were observed in the highest concentration in the upper portion of the dorsal horn (Figure 3f), and they appeared in the medial gray matter as a concentration of delicate immunoreactive varicosities, descending down to the medial part of the ventral horn. At the L5 level a few bundles of very prominent VACHT-immunoreactive fibres running down through the centro-medial part of the ventral horn were observed (Figure 3g).

In general, VACHT immunostaining in comparison with ChAT labelling resulted in pronounced staining of neural processes, while perikarya were found to be stained less intensely.

AChE-histochemistry

The AChE-histochemistry, yielding a brown, granular precipitate, revealed light, moderate and intensely stained neuronal somata and fibres. The AChE-positive neurons were identified in the same spinal regions that were seen to possess both ChAT- and VACHT-immunoreactive cells (Figure 1c). They were distributed in the gray matter of the ventral horn, intermediomedial and intermediolateral nuclei; smaller concentrations of stained cells were also found in the area around the central canal and at the base of the dorsal horn. The general distribution and morphology of AChE-positive perikarya in the studied regions matched well with the morphology and organization of previously visualized ChAT- and VACHT-immunoreactive structures.

Comparison of ChAT-immunocytochemistry and AChE-histochemistry on the same tissue sections

A comparative analysis of the same sections stained one after the other with anti-ChAT antibody and AChE-histochemistry revealed the regionally dependent colocalization of the markers studied. In the ventral horn and intermediolateral nucleus all visualized ChAT-immunostained motoneurons were found to be AChE-positive, and vice versa (Figure 4 a,b and c,d). By contrast, the intermediomedial nucleus showed the incomplete colocalization of the two markers (Figure 4 e,f). While all ChAT-immunoreactive perikarya were AChE-positive, some AChE-stained cells were

found to be ChAT-negative, indicating that the subpopulation of AChE-synthesizing neurons does not express ChAT. The total number of ChAT-stained cells counted in the intermediomedial nucleus in the double method accounted for about 64% of the cells stained for AChE (Table 1).

Moreover, the double staining applied revealed a number of single AChE-positive neurons dispersed throughout the gray matter (Figure 1c). The cells were devoid of ChAT-immunofluorescence.

DISCUSSION

The present investigation revealed the distribution and morphology of cholinergic neurons and fibres in the thoracolumbar and sacral spinal cord of the pig. Within the studied region of the cord prominent concentrations of cholinergic neurons were identified in the gray matter of the ventral horn, in the intermediolateral column (which forms an intermediolateral nucleus), in the intermediomedial nucleus, and individual stained cells were found in lamina X in the area around the central canal and at the base of the dorsal horn. Although the general pattern of the porcine spinal thoracolumbar cholinergic system closely resembles those reported in rat (Houser et al., 1983; Barber et al., 1984), mouse (Chan-Palay et al., 1982), rabbit (Kan et al., 1978), cat (Kimura et al., 1981; Motavkin and Okhotin, 1980) and humans (Muroishi et al., 2000; Oda and Nakanishi, 2000), visible interspecies differences have been revealed.

Depending on the segmental location our investigation revealed cholinergic fibres in laminae I–IV of the dorsal horn of the cord. The presence of cholinergic processes in laminae III–V was described in the rat (Houser et al., 1983; Barber et al., 1984) and confirmed biochemically in the dorsal horn of the humans (Aquilonius et al., 1981). This is in agreement with a report on the mouse (VanderHorst and Ulfhake, 2006) in which the immunoreactivity for VACHT was observed in lamina II of the spinal dorsal horn. On the other hand, the application of three distinct procedures for ChAT-, VACHT-immunocytochemistry and AChE-histochemistry identified the cholinergic nature of the sacral parasympathetic nucleus in the pig. Similarly to our results the cholinergic nature of the nucleus was reported in the rat (Borges and Iversen, 1986), however it was not found in the mouse (VanderHorst and Ulfhake, 2006), indicat-

ing a different neurochemical characteristic of the intermediomedial neurons in the mouse. Moreover, in contrast to findings in the mouse (VanderHorst and Ulfhake, 2006) and guinea pig (Doone et al., 1999) the VACHT- and ChAT-immunoreactive signals were not detected in the lateral collateral pathway (LCP) of the pig. The LCP receives primary afferents from pudendal and pelvic nerves (Nadelhaft and Booth, 1984) supplying the genital organs among others. The observed discrepancies may thus reflect either interspecies differences in the chemical coding of the LCP or neurochemical immaturity of the pathway in the juvenile animals used in this study.

We found that the neuropil of the ventral horn, and to a lesser extent intermediolateral and intermediomedial nuclei, contained both ChAT- and VACHT-positive bouton-like structures, forming even basket-like forms surrounding motoneurons. Morphologically similar ChAT-immunoreactive boutons in contact with the perikarya and dendrites of motor neurons were reported in the ventral horn of the spinal cord of the rat (Houser et al., 1983; Barber et al., 1984; Borges and Iversen, 1986) and were found by electron microscopy to be synaptic terminals (Houser et al., 1983; Connaughton et al., 1986). The terminals denominated as cholinergic C-type were shown (Hellstrom et al., 2003) to be associated with muscarinic receptor type 2 in the postsynaptic membrane. Although the existence of synaptic connections between the bouton-like structures and motoneuronal somata has not been confirmed in the pig yet, it is likely that the motor cholinergic neurons are cholinceptive in nature.

Little information is available on the origin of the cholinergic terminals on the spinal motor neurons; however, ultrastructural and tract-tracing studies in the cat (Cullheim et al., 1977; Lagerback et al., 1981) indicated a possibility that in the spinal cord recurrent axon collaterals of motor neurons make synaptic contacts with the perikarya and proximal dendrites of α -motoneurons. At present, however, the origin of such cholinergic terminals in the porcine spinal cord is unknown and requires further study.

While the topography as well as the morphology of ChAT- and VACHT-immunoreactive neurons in the porcine spinal cord is congruent, the comparison of both stainings revealed peculiar discrepancies concerning the appearance of visualized immunoreactive structures. Indeed, ChAT-immunostaining disclosed intensely immunofluorescent neuronal somata filled with a smoothly distributed immu-

noprecipitate, surrounded by scarce boutons. By contrast, the VAcHT antibody demonstrates a “clump”-like immunoreactive material, filling the interior of the cells which are encircled by numerous, intensely stained boutons. This difference is in agreement with a previous report (Weihe et al., 1996) and reflects different subcellular distribution of acetylcholine-synthesizing enzyme ChAT and acetylcholine transporter protein, VAcHT.

In our study, as revealed by single AChE histochemistry, the AChE-positive neurons occupied the same regions of the studied spinal cord as ChAT- and VAcHT-immunoreactive cells. Consequent double staining disclosed the complete colocalization of ChAT and AChE in the ventral horn and intermediolateral nucleus. In these nuclei our findings closely correlate with the current report of Mis (2005) showing that in the ventral horn of the spinal cord of the rat all neurons expressing mRNAs for AChE were found to be ChAT-positive. On the other hand, the precise correlation between the two cholinergic enzymes in the ventral horn and the intermediolateral nucleus occurs in contrast to the intermediomedial nucleus, where only 64% of AChE-positive cells were found to express ChAT-immunoreactivity. Our results indicate that the relationship between the two cholinergic enzymes, ChAT and AChE, in the porcine spinal cord is complex and may imply different metabolic profiles of AChE-expressing neurons.

REFERENCES

- Aquilonius S.M., Eckernas S.A., Gillberg P.G. (1981): Topographical localization of choline acetyltransferase within the human spinal cord and a comparison with some other species. *Brain Research*, 211, 329–340.
- Arvidsson U., Riedl M., Elde R., Meister B. (1997): Vesicular acetylcholine transporter (VAcHT) protein: a novel and unique marker for cholinergic neurons in the central and peripheral nervous system. *The Journal of Comparative Neurology*, 378, 454–467.
- Baltadzhieva R., Gurevich T., Korczyn A.D. (2005): Autonomic impairment in amyotrophic lateral sclerosis. *Current Opinion in Neurology*, 18, 487–493.
- Barber R.P., Phelps P.E., Houser C.R., Crawford G.D., Salavatterra P.M., Vaughn J.E. (1984): The morphology and distribution of neurons containing choline acetyltransferase in the adult rat spinal cord: an immunocytochemical study. *The Journal of Comparative Neurology*, 229, 329–346.
- Borges L.F., Iversen S.D. (1986): Topography of choline acetyltransferase immunoreactive neurons and fibers in the rat spinal cord. *Brain Research*, 362, 140–148.
- Bruce G., Wainer B.H., Hersh L.B. (1985): Immunoaffinity purification of human choline acetyltransferase: comparison of the brain and placental enzymes. *Journal of Neurochemistry*, 45, 611–620.
- Calka J., Kaleczyc J., Majewski M. (1993): Histochemical demonstration of acetylcholinesterase (AChE) activity in some regions of the hypothalamus in the pig. *Zoologische Jahrbuch für Anatomie*, 123, 187–196.
- Chan-Palay V.A., Engel G., Palay S.L., Wu J.Y. (1982): Synthesizing enzymes for four neuroactive substances in motor neurons and neuromuscular junctions: light and electron microscopic immunocytochemistry. *Proceedings of the National Academy of Sciences of the United States of America*, 79, 6717–6721.
- Connaughton M.J., Priestley V., Sofroniew M.V., Eckenstein F., Cuello A.C. (1986): Inputs to motoneurons in the hypoglossal nucleus of the rat: light and electron microscopic immunocytochemistry for choline acetyltransferase, substance P and enkephalins using monoclonal antibodies. *Neuroscience*, 17, 205–224.
- Cullheim S., Kellerth J.O., Conradi S. (1977): Evidence for direct synaptic interconnections between cat spinal α -motoneurons via the recurrent axon collaterals: a morphological study using intracellular injection of horseradish peroxidase. *Brain Research*, 132, 1–10.
- Davidoff M.S., Galabov P.G., Bergmann M. (1989): The vegetative network in the thoracolumbar spinal cord of the guinea pig: A comparison of the distribution of AChE-enzyme activity and choline acetyltransferase-like immunoreactivity. *Journal für Hirnforschung*, 30, 707–717.
- Doone G.V., Pellisier N., Manchester T., Vizzard M.A. (1999): Distribution of NADPH-d and nNOS-IR in the thoracolumbar and sacrococcygeal spinal cord of the guinea pig. *Journal of the Autonomic Nervous System*, 77, 98–113.
- Fonnum F. (1973): Recent developments in biochemical investigations of cholinergic transmission. *Brain Research*, 62, 497–507.
- Galabov P. (1976): Characteristics of the cholinergic network from the intermediate zone of the thoracic cord of some mammals. *Comptes Rendus de l'Académie Bulgare des Sciences*, 29, 431–434.
- Galabov P., Davidoff M. (1976): On the vegetative network of guinea pig thoracic spinal cord. *Histochemistry*, 47, 247–255.
- German D.C., Bruce G., Hersh L.B. (1985): Immunohistochemical staining of cholinergic neurons in the hu-

- man brain using a polyclonal antibody to human choline acetyltransferase. *Neuroscience Letters*, 61, 1–5.
- Hellstrom J., Oliveira A.L.R., Meister B., Cullheim S. (2003): Large cholinergic nerve terminals on subset of motoneurons and their relation to muscarinic receptor type 2. *The Journal of Comparative Neurology*, 460, 476–486.
- Houser C.R., Crawford G.D., Barber R.P., Salavatterra P.M., Vaughn J.E. (1983): Organization and morphological characteristics of cholinergic neurons: an immunocytochemical study with a monoclonal antibody to choline acetyltransferase. *Brain Research*, 226, 97–119.
- Ichikawa T., Ajiki K., Matsuura J., Misawa H. (1997): Localization of two cholinergic markers, choline acetyltransferase and vesicular acetylcholine transporter in the central nervous system of the rat: *in situ* hybridization histochemistry and immunohistochemistry. *Journal of Chemical Neuroanatomy*, 13, 23–39.
- Kan K.S., Chao L.P., Eng L.F. (1978): Immunohistochemical localization of choline acetyltransferase in rabbit spinal cord and cerebellum. *Brain Research*, 146, 221–229.
- Karnovsky M.J., Roots L. (1964): A 'direct-coloring' thiocholine method for cholinesterase. *The Journal of Histochemistry and Cytochemistry*, 12, 219–221.
- Kimura H., McGeer P.L., Peng J.H., McGeer E.G. (1981): The central cholinergic system studied by choline acetyltransferase immunohistochemistry in the cat. *The Journal of comparative neurology*, 200, 151–201.
- Lagerback P.A., Ronnevi L.O., Cullheim S., Kellerth J.O. (1981): An ultrastructural study of the synaptic contacts of α -motoneurone axon collaterals. I. Contacts in lamina IX and with identified α -motoneurone dendrites in lamina VII. *Brain Research*, 207, 247–266.
- Malatova Z. (1983) Distribution of choline acetyltransferase and acetylcholinesterase in the nervous system of the dog. *Physiologia Bohemoslovaca*, 32, 129–134.
- Miampamba M., Yang H., Sharkey K.A., Tache Y. (2001) Intracisternal TRH analog induces Fos expression in gastric myenteric neurons and glia in conscious rats. *American Journal of Physiology. Gastrointestinal and Liver Physiology*, 280, G979–991.
- Mis K. (2005): Colocalization of acetylcholinesterase, butyrylcholinesterase and choline acetyltransferase in rat spinal cord. *Human & Experimental Toxicology*, 10, 543–545.
- Motavkin P.A., Okhotin V.E. (1980): Histochemistry of choline acetyltransferase in the spinal cord and spinal ganglia of the cat. *Neuroscience and Behavioral Physiology*, 10, 307–310.
- Muroishi Y., Kasashima S., Nakanishi I., Oda Y. (2000): Immunohistochemical and *in situ* hybridization studies of choline acetyltransferase in large motor neurons of the human spinal cord. *Histology and Histopathology*, 15, 689–696.
- Nadelhaft I., Booth A.M. (1984): The location and morphology of preganglionic neurons and the distribution of visceral afferents from the rat pelvic nerve: a horseradish peroxidase study. *The Journal of Comparative Neurology*, 226, 239–245.
- Oda Y. (1999): Choline acetyltransferase: the structure, distribution and pathologic changes in the central nervous system. *Pathology International*, 49, 921–937.
- Oda Y., Nakanishi I. (2000): The distribution of cholinergic neurons in the human central nervous system. *Histology and Histopathology*, 15, 825–834.
- Satoh K., Fibiger H.C. (1985): Distribution of central cholinergic neurons in the baboon (*Papio papio*). I. General morphology. *The Journal of Comparative Neurology*, 236, 197–214.
- Swindle M.M., Moody D.C., Philips L.D. (1992): Swine as Models in Biomedical Research. Iowa State University Press, Ames. 312 pp.
- VanderHorst V.G.J.M., Ulfhake B. (2006): The organization of the brainstem and spinal cord of the mouse: relationships between monoaminergic, cholinergic, and spinal projection systems. *Journal of Chemical Neuroanatomy*, 31, 2–36.
- Weihe E., Tao-Cheng J.H., Schafer M.K., Erickson J.D., Eiden L.E. (1996): Visualization of the vesicular acetylcholine transporter in cholinergic nerve terminals and its targeting to a specific population of small synaptic vesicle. *Proceedings of the National Academy of Sciences of the United States of America*, 93, 3547–3552.

Received: 2008–01–31

Accepted after corrections: 2008–07–24

Corresponding Author:

Prof. Jaroslaw Calka, University of Warmia and Mazury, Faculty of Veterinary Medicine, Department of Functional Morphology, Division of Animal Anatomy, Oczapowskiego 14, 10-719 Olsztyn, Poland
Tel. +89 523 37 33, fax +89 523 49 86, e-mail: calkaj@uwm.edu.pl