Organization of acetylcholine-containing structures in the cranial motor nuclei of the rhombencephalon of the pig

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ABSTRACT: We explored the immunoreactivity of choline acetyltransferase (ChAT) in the cranial nerve motor nuclei of the porcine rhombencephalon to reveal the cholinergic nature of these regions. In our experiments we used an immunohistochemical method for the visualization of all acetylcholine-containing structures. All studied motor nuclei contained ChAT-positive cell bodies and fibres, but the intensity of staining differed between the nuclei. Furthermore, characteristic ChAT-immunoreactive bouton-like structures, which are known to be synaptic terminals of the cholinergic system, were observed in the borders of all studied regions. The localization of ChAT-positive "boutons" in the neuropil of the examined nuclei and their proximity to stained perikarya allowed us to differentiate two groups of motor nuclei in the rhombencephalon of the pig: (a) Nuclei containing ChAT-positive bouton-like structures dispersed in the neuropil, often establishing contacts with the stained cell bodies – motor trigeminal, abducent, facial, ambiguous and hypoglossal nuclei. (b) Nuclei in which characteristic boutons were dispersed among the ChAT-positive cells, but were devoid of any contact with perikarya – dorsal motor nucleus of the vagus nerve. These results provide new data on the porcine central nervous system and could be useful in further experiments on amyotrophic lateral sclerosis (ALS) – the disease that results in the progressive degeneration of motoneurons in the brain and spinal cord.

Keywords: cranial nerve motor nuclei; choline acetyltransferase; immunoreactivity; swine

Acetylcholine is a neurotransmitter that plays a crucial role in the communication between central cholinergic motoneurons. Impairment of the cholinergic system implying the loss of motor perikarya underlies cholinergic disorders e.g. amyotrophic lateral sclerosis or multiple system atrophy (Milonas, 1998; Benarroch et al., 2006). Currently, choline acetyltransferase (ChAT) – the acetylcholine biosynthetic enzyme is generally accepted as a specific marker for the identification of cholinergic neurons in both the central and the peripheral nervous system (Eckenstein and Thoenen, 1982; Furness et al., 1983; Wainer et al., 1984; Kasa, 1986; Schemann et al., 1993, 1995).

Immunohistochemistry by using specific antibodies to ChAT allowed to elucidate the organization of the central cholinergic system (Salvaterra and Vaughn, 1989; Wainer and Mesulam, 1990). Immunocytochemical analyses revealed details concerning the morphology and topography of motor neurons in the brainstem and spinal cord of the rat (Armstrong et al., 1983; Houser et al., 1983; Satoh et al., 1983; Barber et al., 1984; Tatehata et al., 1987; Oh et al., 1992; Borges and Iversen, 2006), cat (Kimura et al., 1981; Jones and Beaudet, 1987; Vincent and Reiner, 1987), monkey (Satoh and Fibiger, 1985) and humans (Mizukawa et al., 1986). Furthermore, the use of the antibody to ChAT allowed to acquire data regarding all ChAT-positive elements located in the cholinergic nuclei, especially bouton-like structures, which are known to be the synaptic terminals of the cholinergic system.

Until now the detailed knowledge of the specific distribution of ChAT-positive structures in the neuropil of the cranial motor nuclei has been incomplete and has been available only for rat

(Ichikawa and Hirata, 1990) and monkey (Ichikawa and Shimizu, 1998). Large farm animals have never been precisely investigated in this respect, and the organization of cranial nerve motor nuclei of the porcine rhombencephalon still remains unknown. Since a selective loss of motoneurons, sparing the dorsal motor nucleus of the vagus, occurs in amyotrophic lateral sclerosis, the explanation of the organization of the cholinergic system in the motor nuclei of the pig, which is an especially valuable species for biomedical research due to its similarities to humans (Swindle et al., 1992), is of particular importance.

In our study we applied a polyclonal antibody against ChAT to examine the morphology and distribution of the ChAT-immunoreactive structures in the cranial nerve motor nuclei of the porcine rhombencephalon.

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 breed (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, *i.v.*) and perfused transcardially with 4% solution of paraformaldehyde in 0.1M phosphate buffer (PB; pH 7.4). After perfusion brains including the medullae oblongatae were collected from all the animals studied. The tissue blocks containing the medullas were 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. Then coronal frozen sections of the medullas were cut in a cryostat at the thickness of 20 μm. Serial sections were mounted on chrome alum-gelatine-coated slides and air-dried.

The sections were subjected to single immunostaining for choline acetyltransferase (ChAT). 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.) at a dilution 1:5 000. The incubation was carried out overnight at room temperature and the next morning the slides were rinsed in PBS (3 × 10 min). Biotinylated secondary antisera (Vector Laboratories Inc.) directed against the host of primary antisera at a dilution 1:400 were 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 a 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), and photographed with a confocal microscope (BIO-RAD).

The specificity of the immunoreaction was confirmed by the omission of primary antiserum as well as by its replacement with normal rabbit serum.

RESULTS

In all studied motor nuclei (trigeminal motor, abducent, facial, ambiguous, hypoglossal, dorsal nucleus of the vagus) ChAT-immunoreactive perikarya and dendrites were observed, however the shape and the size of motoneurons and the intensity of neuronal staining differed between nuclei. Additionally, the neuropil of all examined nuclei contained ChAT-positive bouton-like structures but their appearance, distribution and proximity to motoneurons or dendrites differed between the nuclei.

Motor nucleus of the trigeminal nerve
(Figure 1)

Motoneurons of the nucleus were moderately ChAT-immunoreactive. They were mostly multipolar and triangular neurocytes with large cell bodies from 40 to 80 μm in diameter. The ChAT-positive fibres penetrating among stained perikarya were observed. In the neuropil of the nucleus the ChAT-positive bouton-like structures, discoidal or hemispherical in shape, were detected. Numerous motoneurons were accompanied by ChAT-immunoreactive bouton-like structures surrounding their perikarya. Most of those structures were found to be in contact with ChAT-positive cell bodies.

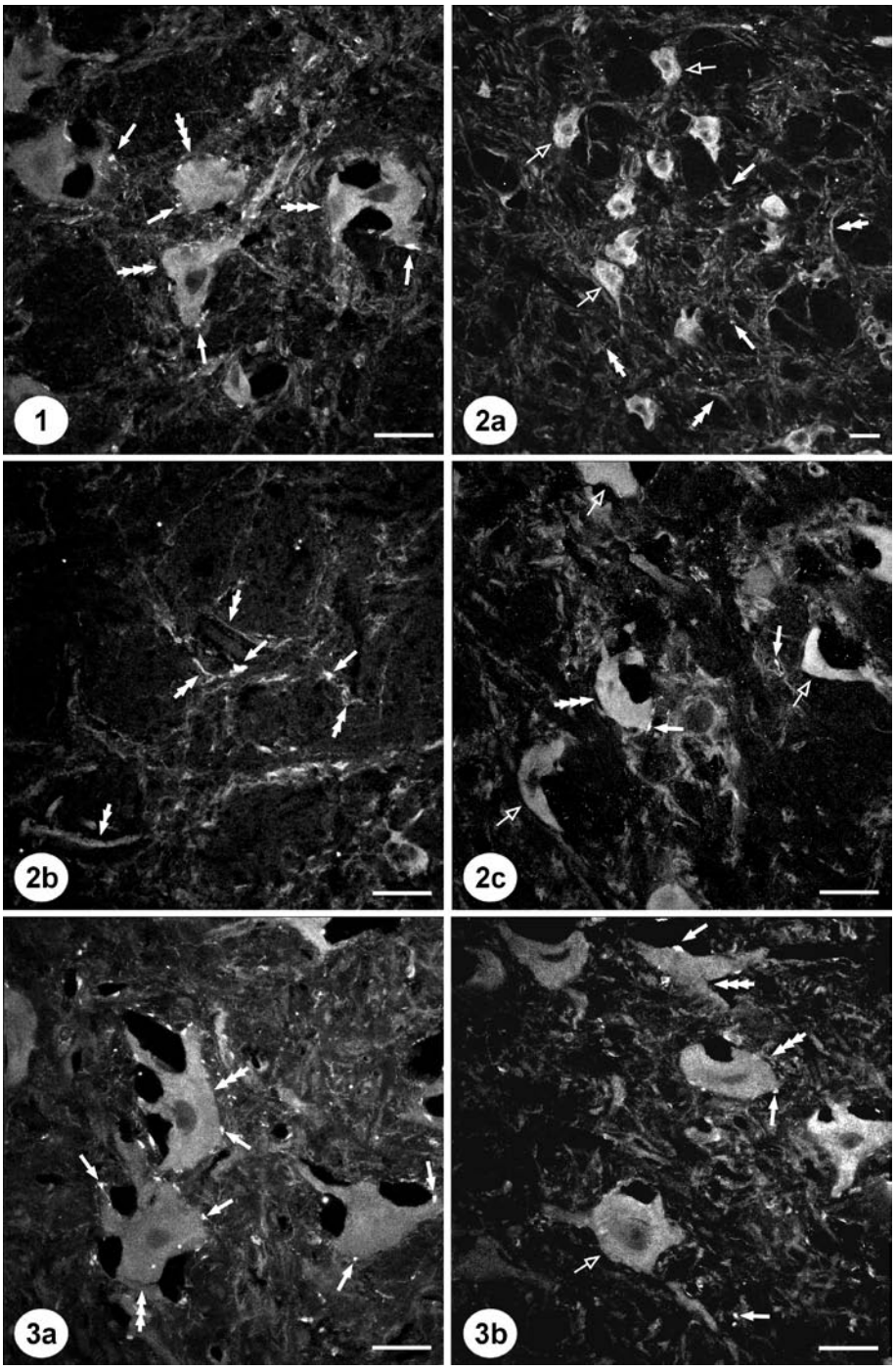


Figure 1. ChAT-positive cell bodies (triple arrows) in the region of the motor nucleus of the trigeminal nerve often connected with ChAT-immunoreactive bouton-like structures (single arrows). Scale bar 40 μm

Figure 2a, b, c. (**a**) Almost all of ChAT-immunoreactive motoneurons (empty arrows) were devoid of any contact with ChAT-positive bouton-like structures (single arrows) and surrounded by numerous ChAT positive fibres (double arrows) in the region of the abducent nucleus. (**b**) Prominent ChAT-positive fibres (double arrows) being in contact with ChAT positive bouton-like structures (single arrows). (**c**) Single cell body (triple arrow) contacted with single ChAT positive bouton-like structure (single arrow) surrounded by perikarya (empty arrows) devoid of any boutons on their surface. Scale bar 40 μm

Figure 3a, b. (**a**) Large, ChAT positive motoneurons (triple arrows) often contacted with many bouton-like structures (single arrows) in the ventral part of the facial nucleus. (**b**) Dorsal part of the nucleus contained also motoneurons (empty arrows) devoid of any contact with ChAT positive "boutons". Scale bar 40 μm

Abducent nucleus (Figure 2a, b, c)

A moderate number of moderately to intensely ChAT-positive cell bodies dispersed in the mesh of prominent ChAT-immunoreactive fibres was found. Most cells were triangular or oval in shape (Figure 2a) and measured about 40 μm in diameter.

In the neuropil of this nucleus a small number of ChAT-positive bouton-like structures were present. The boutons occupying the nuclear neuropil often established contacts with prominent fibres (Figure 2b). Only individual cell bodies appeared to communicate with single ChAT-positive boutons (Figure 2c), while the rest of perikarya were devoid of direct connections.

Facial nucleus (Figure 3a, b)

The light microscopic analysis revealed ChAT-positive cell bodies, fibres as well as bouton-like structures in the region of the nucleus. The morphology and intensity of staining of the motoneurons resembled those of the trigeminal nucleus although the size of the perikarya varied from 40 to 100 μm in diameter. Bouton-like structures were strongly ChAT-immunopositive, hemispherical in shape, and many of them seemed to be in contact with ChAT-immunoreactive neurons.

The number of ChAT-positive structures that looked like to establish direct contacts with the perikarya differed depending on the specific spatial subregional location in the region of the facial nucleus. The motoneurons of ventrolateral and ventromedial subnuclei showed the highest number of abutting boutons. Some of the perikarya of these subnuclei were found to be surrounded by numerous ChAT-positive boutons (Figure 3a). The number of the stained structures surrounding motoneuronal perikarya decreased towards the dorsal part of the nucleus (dorsomedial and dorsolateral subnuclei) (Figure 3b) and reached the minimum level at the central part of the facial nucleus (medial subnucleus).

Ambiguous nucleus (Figure 4)

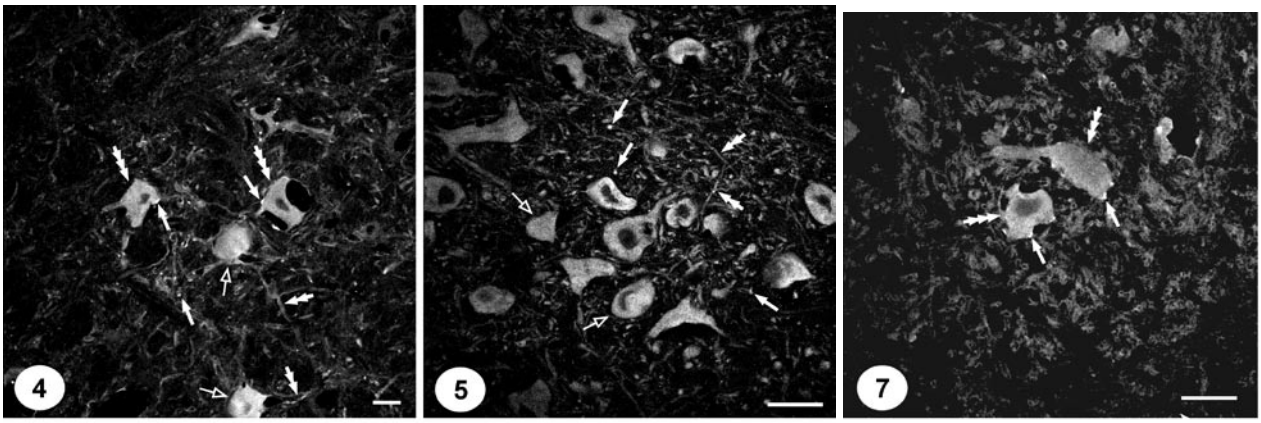
The nucleus characterized a sparse distribution of the ChAT labelled neurons. These cells were moderately to intensely labelled and had oval or multipolar morphology. The diameter of perikarya varied from 40 to 80μm. In addition to cell bodies the bundles of ChAT-immunoreactive processes were observed. In the neuropil of the nucleus numerous ChAT-positive bouton-like structures were found. More than a half of the motoneurons seemed to be in contact with these structures, however perikarya of neurons devoid of contacts were mostly smaller (40–60 μm).

Dorsal nucleus of the vagus nerve (Figure 5 and 6a, b, c)

The light microscopic analysis revealed that the nucleus had a heterogeneous structure depending on the cross section. In the caudal (Figure 6a) and rostral (Figure 6c) part of the nucleus, the ChAT-positive neurons constituted compact groups, round or oval in shape, composed of 15–20 cells. In the middle part of the nucleus, the ChAT-immunoreactive neurons were dispersed and divided into two major groups which were connected together, and the nucleus as a whole was semilunar in shape (Figure 6b). The first group was placed more dorsally near the fourth ventricle, while the second group was located ventrolaterally from the first one. All the stained neurons showed the same, moderate to high fluorescent activity. Most of the cells were oval and triangular in shape with the nucleus situated in the centre. The perikarya measured about 20 to 40 μm. A large number of the ChAT-positive fibres interspersed between ChAT-positive cells was observed in the nucleus. A large number of the ChAT-positive bouton-like structures was found in the matrix of the nucleus. Motoneurons were free from any ChAT-immunoreactive structures on their surface.

Hypoglossal nucleus (Figure 7)

Weak to moderately labelled ChAT-positive motoneurons were present in the area of the nucleus. The cell bodies were medium to large (about 40–60 μm in diameter), multipolar and triangular in shape, with round nuclei situated in the centre of perikarya. Occasionally, ChAT-immunopositive nerve fibres were observed between the stained cell bodies. The fibres were distributed as single processes coursing in the neuropil of the nucleus. Numerous boutons were found to be adjacent to



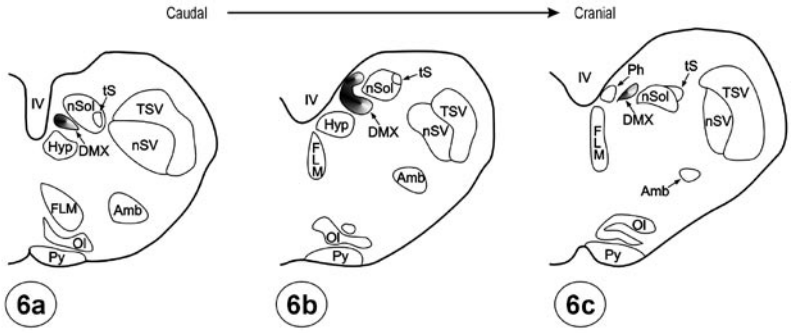


Figure 4. Two types of ChAT-positive motoneurons occurred in an approximately equal number in the region of the ambiguous nucleus – perikarya (triple arrows) being in contact with ChAT immunoreactive bouton-like structures (single arrows), and cell bodies (empty arrows) devoid of any "boutons" on their surface. Many ChAT-positive fibres (double arrows), and bouton-like structures (single arrows) dispersed in the region of the nucleus. Scale bar 40 μm

Figure 5. Oval or triangular ChAT-stained motoneurons (empty arrows) devoid of any contact with ChAT-positive bouton-like structures in the area of the dorsal nucleus of the vagus nerve. All the "boutons" (single arrows) are dispersed in the mesh of numerous fibres (double arrows). Scale bar 40 μm

Figure 6a, b, c. Drawings presenting schematic localization of the dorsal nucleus of the vagus nerve (DMX) on transverse cross-sections planes [(**a**)-caudal, (**b**)-medial, (**c**)-cranial] of the porcine medulla oblongata: nSV = spinal nucleus of the trigeminal nerve; TSV = spinal tract of the trigeminal nerve; IV = fourth ventricle; Py = pyramid; PH = nucleus prepositus hypoglossi; Hyp = nucleus nervi hypoglossi, nSol = nucleus tracti solitari; tS = tractus solitarius; Amb = nucleus ambiguous; Ol = inferior olive; FLM = medial longitudinal fasciculus; the arrow points out the rostral direction

Figure 7. ChAT-positive cell bodies (triple arrows) often being in contact with ChAT-immunoreactive bouton-like structures (single arrows) in the area of the hypoglossal nucleus. Scale bar 40 μm

the nuclear motoneurons although some perikarya were devoid of boutons.

DISCUSSION

Our study provides data on the presence of ChAT-immunoreactive structures in the motor nuclei of the rhombencephalon of the pig. All studied nuclei were found to contain large cholinergic perikarya, neuronal processes and bouton-like structures. The occurrence and morphology of the ChAT-positive motoneurons observed in the porcine rhombencephalon motor nuclei closely resembled those reported in the rat (Armstrong et al., 1983; Houser et al., 1983; Satoh et al., 1983; Oh et al., 1992), mouse (VanderHorst and Ulfhake, 2006), cat (Kimura et al., 1981; Jones and Beaudet, 1987), dog (Malatova, 1983), baboon (Satoh and Fibiger, 1985) and humans (Mizukawa et al., 1986; Oda and Nakanishi, 2000).

The ChAT-positive bouton-like structures are known to be the cholinergic nerve terminals. The majority of the terminals which are located on motoneurons were shown to be C-terminals (Connaughton et al., 1986; Li et al., 1995; Gilmor et al., 1996; Hellstrom et al., 2003). The presence of the ChAT-immunoreactive boutons was already reported in the spinal cord and cranial nerve motor nuclei (Kimura et al., 1981; Houser et al., 1983; Connaughton et al., 1986; Ichikawa et al., 1987; Nagy et al., 1993), while only two reports provided precise information on their spatial location in relation to the cholinergic motoneurons in the rat (Ichikawa and Hirata, 1990) and monkey (Ichikawa and Shimizu, 1998). Our experiment revealed internuclear differences between the ChAT-positive bouton-like structures and motoneurons of the particular motor nuclei of the porcine rhombencephalon. Based on these differences the nuclei might be classified into two main groups. The first group of nuclei is composed of perikarya establishing contacts with the boutons. The trigeminal motor, facial and hypoglossal nuclei constitute a subgroup in which almost all motoneurons have numerous contacts with ChAT-positive bouton-like structures. In the ambiguous nucleus, about a half of the motoneuron population was devoid of the boutons, while in the abducent nucleus only few, individual bouton/perikaryon contacts were observed. Moreover, the number of contacting boutons seemed to depend on the size of the motoneurons. Apparently smaller perikarya of the first group seemed to possess less contacting boutons or were devoid of those structures at all.

The second group includes the dorsal motor nucleus of the vagus (DMX), which contained numerous ChAT-positive boutons, dispersed solely in the mesh of the ChAT-immunofluorescent fibres, but being without any contacts with ChAT-immunoreactive perikarya. Our findings are similar to those reported in monkey (Ichikawa and Shimizu, 1998) but differ insignificantly from results obtained in rat (Ichikawa and Hirata, 1990), in which the whole neuropil of the DMX was free from any CHAT-positive boutons. These similarities between the monkey belonging to primates and the pig – our animal model, may be a further evidence that pigs are an especially valuable species for biomedical research. Our study provides data on the heterogeneous morphology of the motoneurons as well as differential relationship between the neurons and the boutons throughout particular nuclei. Our findings provide an evidence of the complex nature of the motor nuclei in the pig and may imply possible functional diversity of the neurons.

The origin of the ChAT terminals also remains unclear. Hitherto experiments provide different data concerning the origin of cholinergic projections to the motor nuclei of the cranial nerves. According to Woolf and Butcher (1989), in the rat the trigeminal motor, facial and hypoglossal nuclei receive cholinergic projections from the pedunculopontine tegmental nucleus. Fort et al. (1989, 1990) demonstrated that in the cat, cholinergic projections to the trigeminal motor and facial nuclei originate in the medullary reticular formation. Other authors have shown that propriobulbar interneurons of the brainstem reticular formation innervate motoneurons in the trigeminal motor, facial, hypoglossal and ambiguous nuclei in the cat (Holstege and Kuypers, 1977; Holstege et al., 1977) and rat (Travers and Norgren, 1983). Currently, the origin of the cholinergic supply of cholinergic motoneurons in cranial nerve motor nuclei of the pig remains unknown and requires further investigations.

The most interesting data coming from our experiment concern the absence of any ChAT-positive bouton-like structures being in contact with perikarya in DMX. As it is known, in amyotrophic lateral sclerosis, the disease that occurs with an extensive loss of cholinergic motoneurons, the neurons in the dorsal nucleus of the vagus are well preserved (Iwata and Hirano, 1979). The absence of any cholinergic positive terminals contacting perikarya in the DMX of the pig provides an evidence that cholioceptivity may have a relation to the defencelessness of cholinergic motoneurons in amyotrophic lateral sclerosis. Our conclusion correlates with an opinion that a reduction in cholinergic inputs (especially c-boutons) on lower motoneurons may be an early event that precedes the neuronal loss, and could be a reason for cell death (Nagao et al., 1998). Furthermore, abnormalities in the cholinergic innervations of motoneurons were the first sign of the pathologic changes observed in an 80-days-old mouse model of ALS (Burkhard, 2005). Our findings may be useful in further investigations of the disease because the presented data testify pigs, and as it is known, the pig due to its anatomical and embryological similarities to humans is an especially valuable species for biomedical research (Swindle et al., 1992). Our experiment also provides new data on the organization of the central nerv-

ous system of the pig – one of the most popular farm animals.

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