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Sources of porcine *longissimus dorsi* muscle (LDM) innervation as revealed by retrograde neuronal tract-tracing

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Abstract: The aim of the present study was to establish the origin of the motor, autonomic and sensory innervation of the L_1-L_2 segment of the porcine *longissimus dorsi* muscle (LDM), in order to provide morphological basis for further studies focusing on this neural pathway under experimental conditions, *e.g.* phototerapy and/or lateral electrical surface stimulation. To reach the goal of the study, multiple injections of the fluorescent neuronal tracer Fast Blue (FB) were made into the LDM region between the spinal processes of the vertebrae L_1 and L_2. The spinal cord (Th_{13}-S_1 segments) as well as the sensory and autonomic ganglia of interest, *i.e.*, dorsal root (DRG) and sympathetic chain ganglia from corresponding spinal cord levels were collected three weeks later. FB-positive (FB^+) motoneurons were observed exclusively within the *nucleus ventromedialis* at L_1 and L_2 spinal cord level, forming the most ventro-medially arranged cell column within this nucleus. Primary sensory and sympathetic chain neurons were found in appropriate ipsilateral ganglia at Th_{15}-L_3 levels. The vast majority of retrogradely traced neurons (virtually all motoneurons, approximately 76% of sensory and 99.4% of sympathetic chain ganglia neurons) was found at the L_1 and L_2 levels. The morphometric evaluation of FB-labeled DRG neurons showed that the majority of them (approximately 66%) belonged to the class of small-diameter perikarya (10-30 μm in diameter), whereas those of medium size (30-80 μm in diameter) and of large diameter (more than 80 μm) constituted 22.6% and 11.5% of all DRG neurons, respectively. The results of the present study demonstrated that the nerve terminals supplying porcine LDM originated from different levels of the spinal cord, dorsal root and sympathetic chain ganglia. Thus, the study has revealed sources and morphological characteristic of somatic, autonomic and spinal afferent neurons supplying porcine LDM, simultaneously pointing out the characteristic features of their distribution pattern.

Key words: Skeletal muscle - Innervation - Dorsal root ganglia - Sympathetic chain ganglia - Spinal motor nuclei - Retrograde tracing - Pig

Introduction

Improper geometry and/or spatial interrelationships of the vertebrae and/or intervertebral discs, often due to the asymmetrical load [35] or muscle spasticity as a consequence of an upper motor neuron lesion [13], lead to pathological processes resulting in changes of spine equilibrium, manifested by such clinical symptoms like scoliosis (humans, animals) or chronic lordosis (mainly animals). Such deformational complex is composed not only of chronic changes in the architecture of neighboring vertebrae, but also includes profound pathological alternations in ligaments and muscles connecting them, as well as in nerve cells controlling muscle entities affected by the illness. Thus, scoliosis is commonly associated with a variety of neuromuscular disorders including both non-neurally driven myopathies as well as conditions affecting upper and lower motor neurons [6]. Surgical and/or microsurgical correction of such deformations is, even if possible from the anatomical point of view, very complicated and expensive. Therefore, many attempts have been made in order to develop alternative techniques that could be used in less severe

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cases, allowing noninvasive treatment of these defects in humans and animals.

Among different methods used so far, lateral electrical surface stimulation (LESS; *e.g.*, [12]) or chemodenervation by means of botulinum A toxin (BTX) injections (*e.g.* [30]) have been thought to be very promising techniques in the therapy of progressive idiopathic scoliosis in children, even though the former technique has recently been shown to be able to produce adverse side-effects when used improperly [29]. On the other hand, low-power laser therapy (LPLT; for review see [21]) and/or ultrasound stimulation [32] has also been proposed as an alternative/support to surgery, especially when spinal cord or peripheral nerves are damaged [3]. However, while LESS [22] and, even more, LPLT (see below) have been found to evoke beneficial effects when used to stimulate injured tissues and/or organs, the exact mechanism(s) leading to such improvement in the clinical status of treated tissues remains still to be an enigma.

One of the possible explanations of the LESS and/or LPLT mode of action may be the hypothesis that both kinds of energy may influence the functional status of neurons involved in the neural control of the affected tissues. Thus, it is assumed that LPTP can promote and upgrade metabolic processes that result in tissue repair [5], in this way leading often to clinically significant enhancement of repair processes in various tissues and organs, *e.g.*, bones [15], cartilage [23], muscles [2], ligaments [14], skin [1] and neural structures, like peripheral nerves (for reviews see [21]), spinal cord [37] and even cultured cortical neurons, by inducing formation and outgrowth of their neurites [43].

The nerve supply of skeletal muscles is complex, comprising of axons of α- and γ-motoneurons located in the ventral spinal horn, efferent terminals of paravertebral sympathetic chain neurons (not only controlling the intramuscular blood vessels, but exerting a neurotrophic effects) and afferent processes of dorsal root ganglia neurons. Hence, to be able to evaluate the effect(s) of LPTP, LESS or BTX on skeletal muscle innervation pattern, the sources of nerve terminals contributing to this nerve pathway have first to be established in detail in an animal model.

To reach this goal, we decided to use the retrograde neuronal tract tracing (by applying fluorescent tracer Fast Blue) from the lumbar portion (L_1-L_2) of the pig *longissimus dorsi* muscle to appropriate centers in the spinal cord, dorsal root sensory ganglia and ganglia of the sympathetic chain.

Materials and methods

The experiments were carried out on three juvenile pigs (approximately 20 kg body weight) of the Large White Polish race obtained from a commercial fattening farm in Trekusek, Poland. The animals were housed and treated in accordance with the rules approved by the local Ethics Commission (conforming to principles of Laboratory Animal Care, NIH publication no. 86-23 , revised in 1985).

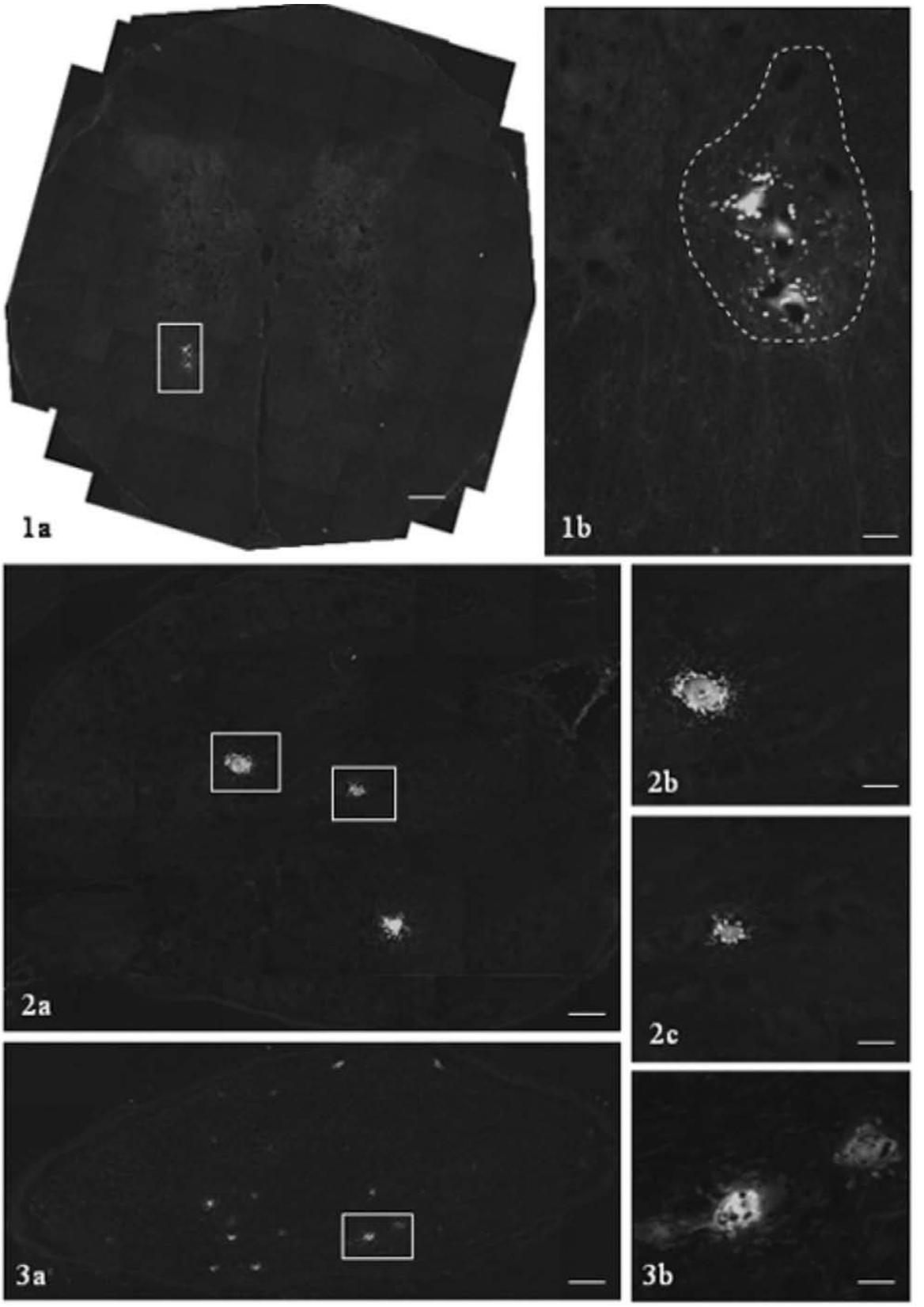
Surgery was performed under fractionated thiobarbital (Thiopenthal, Sandoz, Austria; 20 mg/kg b.w., i.v.) anesthesia. Prior to administration (30 min) of the main anesthetic, the pigs were pretreated with atropine sulphate (Polfa, Poland; 0.04 mg/kg b.w., s.c.) and azaperone (Stressnil, Janssen Pharmaceutica, Belgium; 2.0 mg/kg b.w., i.m.). The retrograde fluorescent tracer Fast Blue (FB; Dr. K. Illing GmbH, Gross-Umstadt, Germany) was injected into the segment of the right *longissimus dorsi* muscle located between spinal processes of the L_1 and L_2 vertebrae. To avoid the labeling of skin-projecting sensory and sympathetic neurons, full-thickness skin incisions (one paralleling the body long axis and situated 3 cm laterally to the midline formed by spinal processes, as well as two transverse, at Th_{15} and L_3 spinal processes) were made and the mobilized skin flap was gently reflected medially. A total volume of 30 µl of 2% aqueous dye solution was injected into the exposed muscle segment using a Hamilton syringe with a 26-gauge needle. Multiple (n=30, 1 µl each) injections were made into the muscle in a chessboard-like manner, any traces of the FB were carefully swapped out and the skin wound was sutured.

After 3 weeks, animals were re-anaesthetized and transcardially perfused with 4% buffered paraformaldehyde (pH 7.4). Subsequently, Th_{12}-L_6 spinal cord segments with corresponding bilateral DRG as well as sympathetic chain ganglia were collected. The tissues were postfixed by immersion in the same fixative for 20 min, then washed with phosphate buffer (pH 7.4) for three days and finally transferred to and stored in 18% buffered (pH 7.4) sucrose solution until further processing. Spinal cord neuromeres and sensory as well as sympathetic ganglia were cut into 12- (spinal cord; transverse sections) or 10-µm-thick cryostat serial sections (dorsal root and sympathetic chain ganglia; longitudinal sections). To determine the relative number of FB-positive cells, the neurons were counted in every eight (spinal cord) or fifth (ganglia) section, in order to avoid double counting. Only neurons with clearly visible nucleus were considered. As spinal sensory neurons are usually oval in shape, to determine to which class (*i.e.*, small, medium-sized or large perikarya) the retrogradely labeled DRGs neurons belonged, their mean diameter was estimated by the use of following equation: mean diameter = (minimal diameter + maximal diameter)/2. Data were pooled from all animals and expressed as means ± SEM. The sections were viewed under an Olympus BX51 fluorescence microscope equipped with a barrier filter for FB. Microphotographs were acquired with a CCD camera connected to a PC equipped with AnalySIS image analysis software (ver. 3.2; Soft Imaging System GmbH, Münster, Germany).

Results

Retrogradely labeled neurons supplying the studied LDM segment were observed exclusively in ventral spinal horn as well as in sensory and sympathetic ganglia ipsilateral to the site of FB injections.

Fig. 1. Low magnification fluorescence microphotograph (montage) of the transverse section of the porcine spinal cord at the L_1 neuromere (**a**). Region marked by the rectangle in (**a**) is shown at higher magnification in (**b**). Doted line marks the ventromedial nucleus of the ventral spinal horn, where the LDM-projecting motoneurons were located. Scale bar: a - 3200 µm, b - 100 µm. **Fig. 2.** Low magnification fluorescence microphotograph (montage) of the longitudinal section of the porcine L_2 dorsal root ganglion (**a**). Higher magnification views of afferent neurons marked by rectangles are shown in **b** and **c**, respectively. Scale bar: a - 800 µm, b and c - 100 µm. **Fig. 3.** Low magnification fluorescence microphotograph (montage) of the longitudinal section of the porcine sympathetic chain ganglion L_1 (**a**). Higher magnification micrograph of two retrogradely labeled postganglionic neurons marked by the rectangle is shown in **b**. Scale bar: a - 1000 µm, b - 50 µm.


1a
1b
2a
2b
2c
3a
3b

The relative number of FB^+ motoneurons found in the porcine spinal cord amounted to 339 ± 43 per animal. These neurons were found exclusively in the ventromedial nucleus of the ventral horn (Fig. 1a), forming a loose cell column spreading over the whole length of L_1 and L_2 spinal cord segments. On transverse spinal cord sections, these neurons formed a discrete cluster composed of 2-6 neurons per section, located at the border of the ventralmost part of the spinal motor horn (Fig. 1b). Of all labeled LDM motoneurons, approximately 43.0 ± 14% were found at L_1 spinal cord level, while 57.0 ± 14% of these cells were disclosed in the L_2 segment (Fig. 4a).

The relative number of FB^+ DRG neurons found in studied animals amounted to 233 ± 31. Some retrogradely labeled sensory perikarya were found from Th_{15}-L_3 (Fig. 4b), but the vast majority was located within the L_1 and L_2 DRG, comprising 76.2 ± 4 .3% of all FB^+ neurons (Fig. 2a). Of all the FB^+ sensory neurons found, the most numerous subpopulation (65.9 ± 8.6%) was composed of small-sized neurons (10-30 μm in diameter; Fig. 2c), while the medium-sized (30-80 μm) cells comprised 22.6 ± 2.9% and the perikarya of the largest diameter (over 80 μm) were the least numerous and constituted 11.5 ± 1.5% of all labeled neurons (Fig. 2b).

The relative number of FB^+ sympathetic chain ganglia neurons amounted to 1320 ± 142. FB^+ sympathetic neurons were found from Th_{15} to L_3 sympathetic chain ganglia, but the overwhelming majority of them were grouped in L_1 and L_2 ganglia (78.1 ± 8.4% and 20.8 ± 8.6% of all FB^+ sympathetic chain ganglia neurons, respectively; Fig. 4c). The vast majority of these cells belonged to the subset of spindle-shaped, relatively small neurons that were more often observed in the peripheral ganglionic regions, while the larger FB^+ cells were relatively more frequently found in the central areas of the ganglia. However, somatotopic distribution of these neurons within the ganglia studied was not observed (Fig. 3a).

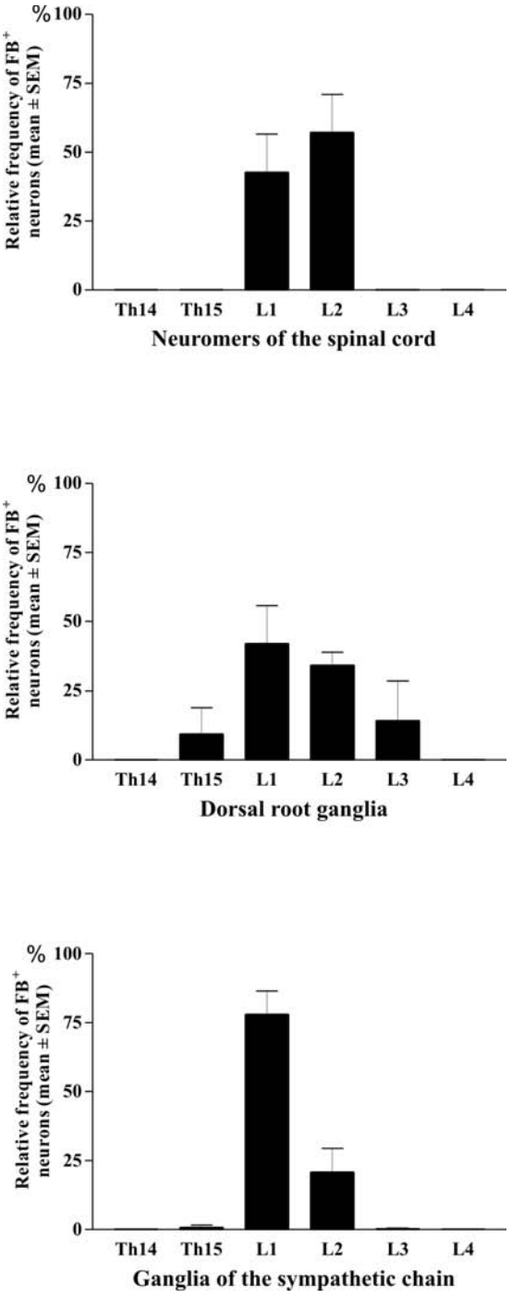


Fig. 4. Histograms showing the distribution pattern and relative frequency (mean ± SEM) of retrogradely labeled motoneurons (**a**), spinal sensory (**b**) and sympathetic chain ganglia neurons (**c**) supplying the studied segment of the LDM in the pig three weeks after application of the tracer.

Discussion

Results of the present study demonstrate the multi-focal origin of somatic, autonomic and sensory nerve fibers supplying the LDM of the pig, giving the morphological background for further studies concerning the neurochemical organization of this nerve pathway.

Numerous studies have concerned the origin of motor fibers supplying the epaxial [8, 24, 31, 39] and hypaxial [16, 39] muscles of the back [8, 16, 24, 31, 39] and tail [28, 41], as well as those of the hind- or forelimb [7, 9, 27, 34, 39, 42], abdominal wall [16, 24] and the pelvic floor [18, 39]. However, only in four species the pattern of motoneuron localization has been studied in detail by means of a very accurate technique of the retrograde transport of a marker from intramuscular nerve endings supplying the particular muscle to their parent cell bodies: monkey [27, 40], hamster [16, 18], cat [24, 31, 39, 41, 42] and rat [7, 8, 28, 34]. Careful analysis of these reports revealed that motoneurons supplying the particular muscle entities were distributed in a topographically specific manner within the ventral

horn (cf. [39, 42]), forming distinct subnuclei (rather "subcolumns", when the whole extent of the ventromedial or ventrolateral cell columns of the neuromeres under study were considered, as described previously in the rat [34]). The distribution pattern of retrogradely labeled MLD motoneurons in the pig is in line with those described for perikarya innervating the lateral *longissimus* and *quadratus lumborum* muscle in hamster [17] and the extensors of the back and tail (*i.e.* medial *longissimus* and lumbar *multifidi*) in cat [24, 39]. Furthermore, such arrangement of FB^+ neurons is also in accordance with the hypothesis that the medially located motoneurons send their axons to the axial muscles by the dorsal rami of the spinal nerves, while the cells of the ventrolateral spinal motor nuclei utilize the ventral rami to reach their targets located within the abdominal wall, hind leg or the pelvic floor [38].

We have observed that the motoneurons innervating the "L_1 segment" of the porcine MLD were located exclusively in two neuromeres, *i.e.*, L_1 and L_2, however, they were distributed along their whole length. This is in line with the study of Izumi and Kida [26], who have demonstrated that segments of the spinal nerves supplying a particular target (muscle) exactly indicate the segmental levels of the supplying motoneurons and also suggest the segments of somites where primordial cells of the muscle were located. Furthermore, as the regression of polyneural innervation in postural muscles appears to be linked to the transition from the immature into the adult-like patterns of postural control [25], *i.e.* one muscle fiber is controlled by only one motoneuron, it may be judged from the number of retrogradely labeled motoneurons observed in the present study that this supraspinal muscle in the pig is composed of slow muscle fibers with a high resistance against fatigue (typical for muscles with strong postural functions) [25].

Considering the diameters of perikarya, the retrograde tracing technique revealed that the spinal sensory neurons innervating the studied segment of porcine MLD belonged to all three classes of primary afferent cells. Of them, the most numerous subset (approximately two-third of all retrogradely labeled DRG cells) was composed of small-sized cells, most probably involved in the conveyance of noci- and (at least in part) the proprioceptive modalities to the spinal cord. The medium-sized and the large afferent neurons formed probably the mechanoceptor/vascular pool for the studied MLD segment (it is assumable, that dendrites of these neurons were distributed mainly to the small blood vessels of skeletal muscles, as in the case of the guinea pig DRG neurons [20]). The majority of MLD sensory neurons (approximately 76%) was found in L_1 and L_2 DRG, creating in this way a "L_1-MLD-segment-related" sensory centre. The existence of such a "centre" correspond well with data obtained in the rat [7, 36], cattle [9] and cat [33], where a similar arrangement of sensory neurons supplying the hindlimb [7, 9, 33] and lower back muscle [36] were observed.

FB-positive sympathetic neurons were observed from Th_{15} to L_3 ganglia of the sympathetic trunk, being most numerous in the L_1 and L_2 ganglia. In contrast to the afferent DRG neurons, which were not strictly limited to L_1 and L_2 ganglia, but their moderate number was also present in Th_{15} and L_3 ganglia, virtually all the sympathetic trunk neurons (more than 98%) were found in L_1 and L_2 ganglia. Thus, the existence of a "sympathetic L_1-MLD-segment-related" center may also be suggested. This is incongruent with the report of Daniels *et al.* [10] who reported a more widespread distribution (from approximately L_3 to S_1 ganglia) of sympathetic epaxial muscle-projecting neurons in the rat after single injections of retrograde tracer in the L_5 segment of the muscle studied. Hence, a species-specific organization of the sources of sympathetic efferent projection to the epaxial musculature in rodents and larger animals has to been taken into consideration, especially when the amount of tracer injected (3 µl in the rat vs. 30 µl in the pig), the volume of the treated muscle and the size of the animals were regarded.

Retrogradely labeled sympathetic neurons were evenly distributed throughout the ganglia of studied pigs, in this respect resembling the distribution pattern of epaxial muscle-projecting postganglionic neurons in the rat [10]. As may be judged from their size and intraganglionic distribution pattern, these neurons belong most probably to the vasoconstrictory (smaller cells) and vasodilatory (larger, probably sympathetic cholinergic) sympathetic pathway, as postulated in rodents for hindlimb-projecting sympathetic chain ganglia neurons [4, 11, 19].

In conclusion, this study revealed a complex pattern of epaxial muscle innervation in the pig, providing simultaneously a detailed description of MLD-projecting motoneuron organization within the spinal cord, as well as of the intra- and interganglionic distribution pattern of sensory and sympathetic neurons involved in this nerve pathway.

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