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Sex Differences in the Number and Size of Motoneurons Innervating Rat Medial Gastrocnemius Muscle

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Summary

The sex differences in the number and morphometric parameters of motoneurons in motor nuclei are poorly known. The aim of this study was to determine the differences in the number and size of alpha and gamma motoneurons of the medial gastrocnemius (MG) muscle in male and female Wistar rats. Retrogradely labelled cell bodies of motoneurons of 6 months old animals were studied following a bath of the proximal stump of the transected MG nerve in a horseradish peroxidase solution. The number and soma diameters of male and female MG motoneurons were determined from serial microscopic images of sections. The weight of the brain and spinal cord was on average 17% higher in males than in females. The mean number of motoneurons was 13% higher in males than in females and amounted to 94 and 83 motoneurons, respectively. In each case, the average soma diameters and cross-section areas of motoneurons in motor nucleus were distributed bimodally: motoneurons smaller than 27.5 μm in diameter were recognized as gamma and greater ones as alpha motoneurons. In males, the motor nucleus contained on the average 66 alpha motoneurons, whereas in females, 56 alpha motoneurons, that is the mean number of alpha motoneurons was 17% higher in males. Moreover, the soma diameters of gamma and alpha motoneurons were significantly bigger in males and the difference amounted 9 and 6%, respectively. It is concluded that the number as well as size of alpha and size of gamma motoneurons in the MG motor nucleus are greater in males.

Introduction

The dimorphism of mammalians concerns mass of skeletal muscles and body, whereas there is a limited data concerning sex differences in morphology of the brain and spinal cord. Analyses of the morphometric parameters of skeletal muscles and their fibres in several species of animals and in humans revealed some sex differences in the muscle mass, the cross-section area of muscles, the total number of muscle fibres (higher in males) and the types of composition of muscle fibres (Ariano et al., 1973; Hitomi et al., 2005; Johnson et al., 1973; Mierzejewska-Krzyżowska et al., 2011, 2012). Our earlier results concerning differences of four rat hindlimb muscles (medial gastrocnemius, soleus, tibialis cranialis and flexor digitorum brevis) indicated the biggest sex differences in heavy myosin content in the slow soleus (Drzymała-Celichowska et al., 2012), whereas the total number of muscle fibres was most markedly different in the fast medial gastrocnemius and the mean diameters of muscle fibres differed most significantly in the tibialis cranialis (Mierzejewska-Krzyżowska et al., 2012).

Although numerous studies analysed changes of age-related properties of motoneurons (Ishihara and Araki, 1988; Hashizume and Kanda, 1995; Hashizume et al., 1988; Kanda and Hashizume, 1998), only few papers described sex-related differences of these neurons. Leslie et al. (1991) revealed sex differences in the muscle mass, muscle fibres and motoneuron size in the rat fast flexor digitorum brevis, one of the plantar foot muscles. The

muscle and motoneurons were larger in adult males than in females, and the differences amounted to 44% for muscle mass, 30% for muscle fibres size and 10% for the cross-section area of motoneurons. Interestingly, the authors did not observe the effects of castration in adult males or androgen treatment in adult females on the target muscle size, but the cross-section area of motoneurons increased in androgen-treated females. Moreover, in males, they found no significant effects of castration on motoneuron soma size.

The motor unit, the smallest functional unit of skeletal muscles is composed of one alpha motoneuron and a group of muscle fibres that it innervates. Previous studies from our laboratory aimed at determining the properties of motor units of the MG muscle of male and female rats revealed numerous differences between their motor unit properties (Celichowski and Drzymała, 2006; Celichowski and Drzymała-Celichowska, 2007). It was found that male muscles, which had a bigger mass, were composed of a greater number of motor units, and the contractile properties of the units were different. In males, the motor units had greater force and longer contraction time, and the muscles contained more fast fatigable and less slow motor units than in females. Slow-type motor units are innervated by small motoneurons (Burke, 1981), and in males, they formed 14% of the population, whereas in females 26% (Celichowski and Drzymała, 2006) what suggests that at least due to this reason, the mean size of motoneurons in males is bigger. Lastly, we have found that the innervation ratios were higher for motor units in males (Mierzejewska-Krzyżowska et al., 2011), and this observation also suggests that their motoneurons have bigger soma and axon diameters. In another series of experiments, employing the electrophysiological method of motor unit number estimation based on a comparison of the mean motor unit force against the muscle force, it was shown that the rat MG was composed of 57 motor units in males and 52 in females (Celichowski and Drzymała-Celichowska, 2007). However, it was later revealed that the forces of motor units in this muscle sum up in a non-linear way, and the force summation is less than linear (Drzymała-Celichowska et al., 2010), which might induce an underestimation of motor unit number with the above method.

Innervation of skeletal muscles was described in numerous studies (Belkheyar et al., 2005; Elgafy et al., 2002; Nazzi et al., 2009; Vanden Noven et al., 1994). The fibres of skeletal muscles are innervated by motoneurons distributed within the ventral horn of the spinal cord or the brainstem; a group of motoneurons of one muscle is called the motor nucleus. Two basic types of motoneurons, alpha and gamma, innervate extrafusal and intrafusal muscle fibres, respectively (Burke, 1981). The size, number of motoneurons and organization of the motor nucleus can be studied using labelling with horseradish peroxidase (HRP) alone (Strick et al., 1976; Burke et al., 1977; Richmond et al., 1978; Illert et al., 1982) or HRP conjugated with wheat germ agglutinin (Swett et al., 1986) as well as with the cholera toxin (McClung et al., 2001). This method makes possible the identification of labelled neurons as smaller (gamma) and larger (alpha) motoneurons due to the bimodal distribution of the size of stained neurons innervating the hindlimb muscles observed in the rat and the cat (Burke et al., 1977; Peyronnard and Charron, 1983; Hashizume et al., 1988).

The present study will investigate motoneurons in male and female rats. They will be labelled directly using the method of retrograde axonal transport of HRP, then the total number of labelled motoneurons will be calculated, and finally, they will be divided into alpha and gamma types on the basis of size differences. The ultimate purpose is to determine sex differences in the number and size of alpha and gamma motoneurons in the motor nucleus of the rat MG. The new data concerning sex differences in motor innervation of skeletal muscles will enrich basic neuroscience knowledge and increase our understanding of differences in motor control processes in males and females.

Materials and Methods

Animals

This study presents the data obtained from 16 Wistar rats, including eight males (body weight 420–530 g) and eight females (body weight 210–320 g), all aged 6 months. The rats were fed a standard laboratory diet *ad libitum* (Labofeed B, Poland) and had free access to tap water. They were maintained in cages in an air-conditioned room with a 12:12 h dark–light cycle and a constant temperature of 21 ± 1°C. Experiments were performed in accordance with the European Union and Polish Law on Animal Protection and approved by the Local Bioethics Committee.

Experimental procedure

During a surgery, animals were kept under pentobarbital anaesthesia (sodium pentobarbital, initial dose of 60 mg/kg i.p., supplemented with additional doses of 10 mg/kg when required). The depth of anaesthesia was verified by assessing withdrawal and pinna reflexes. The nerve branch to the MG muscle was isolated from surrounding tissues and was cut near the entry into the muscle. The end of the proximal stump of the cut nerve branch was placed in a little hollow in a small polystyrene plate. About 2 μl of a fresh solution

of 30% HRP (Sigma type VI) in sterile saline was delivered into the hollow with the nerve using a Hamilton microsyringe. After 90 min of the nerve bath, the muscles and skin were closed with sutures. Afterwards, the rats were transported back to their home cages.

Perfusion and isolation of the central nervous system

The animals survived for 72 h and then were deeply re-anesthetized and sacrificed by transcardiac perfusion with 500 ml of warm phosphate-buffered saline mixed with heparin (37°C, up to 15 min), followed immediately by 500 ml of a cold fixative mixture (1.25% glutaraldehyde and 1.0% paraformaldehyde in 0.2 M phosphate buffer, pH 7.4, 4°C, up to 25 min) and subsequently with 500 ml of phosphate buffer containing 10% sucrose at 4°C.

After the perfusion, the central nervous system (CNS), that is, the brain and spinal cord, was exposed, removed and weighed, then the lumbo-sacral segments (L3-S1) of the spinal cord were put for 18–22 h in 30% sucrose buffer at 4°C for cryoprotection.

Histological technique

The lumbo-sacral fragment of the spinal cord, including the L4-L6 segments, was placed in a freezing microtome as a single block and cut into 90-μm-thick serial cross-sections, each collected separately in dishes containing 0.1 M phosphate buffer. To make HRP activity visible, all sections were processed histochemically with the chromogen tetramethylbenzidine using Mesulam's (1978) procedure. After processing, sections were mounted on chrom-alum-gelatinized slides in serial order and counterstained with a neutral red solution, cleared in xylene and coverslipped with DePeX.

Appearance of labelled motoneurons

For identification of labelled motoneurons, all mounted sections were examined under bright-field microscope (Jenaval, Carl Zeiss, Germany) with magnitudes 10× and 25× using Sony CCD-Iris camera. The digitized images were then transferred to the MultiScanBase System for Windows (Computer Scanning System, version 14.02, Warsaw, Poland). In each section, the number of labelled motoneurons was determined to calculate the total number of motoneurons in the MG motor nucleus. The HRP-labelled cells were counted only when the nucleus (i.e. the central non-labelled region of the pericaryon) was visible (Hashizume et al., 1988). This approach minimized the possibility of counting the same neurons twice at the border of sections. The cross-section area and average soma diameter of motoneurons were measured by drawing the outline of an HRP-filled cell body and counted using a computer program. According to the criteria of Burke et al. (1982), the average diameter was a one-half of the sum of the measured maximum and minimum orthogonal diameters.

Statistical analysis

The Mann–Whitney *U*-test was applied to evaluate the significance of the studied sex differences (Statistica program, StatSoft, version 8.0, Cracow, Poland).

Results

The mass of the CNS was approximately 17% higher in males than in females (difference significant, Table 1). However, the ratio of the CNS mass to the body mass was significantly higher in females (Table 1).

Table 1. Mean values, standard deviations and variability ranges for the body mass, the central nervous system (CNS) mass, the ratio of CNS mass to body mass and the number of motoneurons in motor nuclei of MG muscle in male and female rats

			Number of motoneurons in MG motor nucleus			Percentage of motoneurons in motor nucleus	
Body mass (g)	CNS mass (g)	CNS mass /body mass (%)	γ+α	γ	α	γ	α
Females (n = 8)							
251 ± 44	2.44 ± 0.18	0.99 ± 0.15	83.7 ± 7.8	27.6 ± 3.9	56.1 ± 7.6	33.0	67.0
210–320	2.10–2.63	0.78–1.16	75–93	22–31	46–66	26–40	60–74
Males (n = 8)							
474 ± 31.1	2.85 ± 0.16	0.60 ± 0.09	94.6 ± 8.5	28.7 ± 6.6	65.8 ± 7.3	30.1	69.9
420–530	2.58–3.03	0.54–0.68	85–109	22–40	59–78	22–38	62–78
**	**	***	*	n.s.	***	n.s.	n.s.

The significance of differences between male and female properties is indicated by asterisks, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, non significant, $P > 0.05$, Mann–Whitney *U*-test.

Examples of microscopic images of retrogradely labelled MG motoneurons from the middle part of the motor nuclei in a female (a) and a male (b) are shown in Fig. 1

Figure 2 shows the bimodal distribution of the diameters and cross-section areas of motoneuronal body cells as a basis for the division of the studied population into alpha- and gamma-size motoneurons. The threshold value of diameters for the alpha/gamma division was 27.5 μm, and there was no sex-related difference in the threshold value. Examples of alpha and gamma motoneurons are indicated by arrows in Fig. 1. The number and proportion of alpha and gamma motoneurons in the MG motor nuclei of female and male rats are summarized in Table 1. The overall number of alpha and gamma motoneurons in a studied motor nucleus was significantly higher in males; the difference amounted to about 13%.

The mean number of gamma motoneurons in the MG motor nucleus was 4% higher in males, and the difference was not significant (Table 1). The mean number of larger alpha motoneurons was 17% higher in males, and this difference was significant (Table 1). Gamma motoneurons accounted for a mean of 33 and 30% of the total studied number of MG motoneurons in females and males, respectively, whereas alpha motoneurons, for 67 and 70%, respectively (Table 1).

The motoneurons in the studied motor nucleus were significantly greater in males than in females, and the difference was approximately 8% (Table 2), whereas the differences in diameters of alpha and gamma motoneurons compared separately amounted to 6 and 9%, respectively (Table 2). The two studied properties, the diameter and the cross-section area, strongly correlated with each other both in females and in males (Fig. 3a,b).

To analyse the motor innervation of the studied muscle in more detail, the mean values of the muscle mass for male and female rats (1110 and 660 mg, respectively; Mierzejewska-Krzyżowska et al., 2011) were divided by the mean number of alpha motoneurons noted in the present study (66 and 56 motoneurons, respectively). The analysis

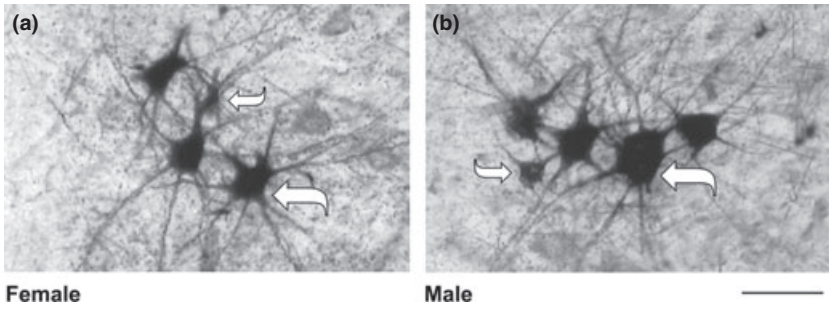

Fig. 1. Photomicrographs of transverse sections through the MG motor nucleus of a female (a) and a male (b) rat taken in the middle part of the nucleus. For each photograph, one alpha and one gamma motoneurons are indicated by a longer and a shorter arrow, respectively. The scale bar represents 70 μm.

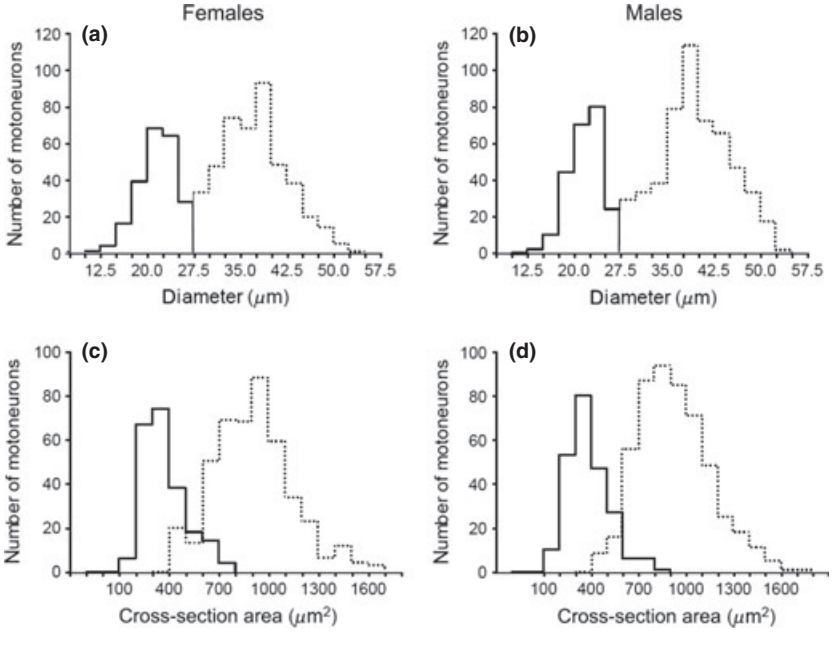

Fig. 2. Distribution of the average soma diameters (a, b) and cross-section areas (c, d) for motoneurons of female (left) and male (right) rats. Note the bimodal distribution of diameters in both motoneuronal populations enabling the division of the motoneurons into two types, gamma (left peak in histograms, solid lines) and alpha (right peak in histograms, dotted lines).

Table 2. Mean values, standard deviations and variability ranges for the diameter and the cross-section area of motoneurons in motor nuclei of MG muscle of male and female rats. The mean values were calculated for the total number of HRP labeled cells

Motoneuron diameter (μm)			Cross-section area of motoneuron ($\mu m^{2)}$		
γ+α	γ	α	γ+α	γ	α
Females					
(n = 661)	(n = 220)	(n = 441)	(n = 661)	(n = 220)	(n = 441)
32.18 ± 8.78	21.63 ± 2.98	37.44 ± 5.28	718.5 ± 313.7	376.5 ± 127.2	879.5 ± 231.6
11.57–53.54	11.57–26.52	27.66–53.54	159.8–1695.0	159.8–779.4	408.6–1695.0
Males					
(n = 757)	(n = 230)	(n = 527)	(n = 757)	(n = 230)	(n = 527)
34.29 ± 9.30	23.55 ± 2.98	39.51 ± 5.57	767.3 ± 316.3	392.2 ± 128.6	938.2 ± 219.8
12.57–53.37	12.57–27.44	27.91–53.37	119.0–1794.8	119.0–804.5	391.1–1794.8
***	***	***	***	*	***

The significance of differences between male and female properties is indicated by asterisks, ***P < 0.001, *P < 0.05, Mann-Whitney U-test.

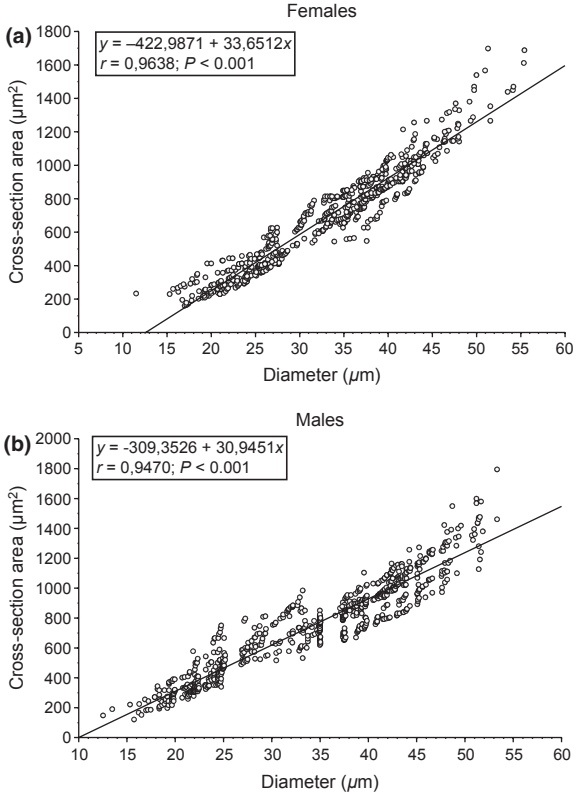

Fig. 3. Correlations between the motoneuronal diameters and cross-section areas for female (a) and male (b) rats. Regression lines, correlation coefficients and the significance level are giver on the plots. The vertical line indicates the border value for the gamma–alpha division of motoneurons.

helped to establish the mean weight of one motor unit and revealed the following sex differences:

Males: 1110 mg/66 motoneurons = 16.81 mg of muscle mass per alpha motoneuron;

Females: 660 mg/56 motoneurons = 11.78 mg of muscle mass per alpha motoneuron.

Discussion

In the present study, the number and size of motoneurons in the motor nucleus of the MG were analysed. We found that the number of motoneurons was significantly higher, and their size was bigger in males than in females.

Efficiency of HRP labelling

In a study of the number and size of motoneurons in the motor nucleus with the use of the retrograde axonal transport method, one can employ different tracers (fluorescent compounds and HRP) and various ways of their application (injections into a muscle or intact nerve and immersion of a cut nerve into a tracer solution). For the purposes of the present study, the use of HRP seemed to be the most appropriate as appeared from a comparison of the labelling efficiency of this tracer with fluorescent ones (Illert et al., 1982). In addition, the application of HRP into a cut nerve is more advantageous because all axons are equally subjected to the tracer, whereas in cases of nerve or muscle injections, the exposure of axons to HRP is less complete. Moreover, several articles indicate that retrograde perikaryal labelling with HRP occurs more readily and completely when the enzyme enters an injured axon than when its uptake is based exclusively on endocytosis (Mesulam, 1982). In pilot experiments with four male rats (not analysed here), we used Fast Blue as a retrograde fluorescent tracer. In each case, the number of all labelled motoneurons was below 70, which is considerably lower than the number obtained in the present study (mean number 94). This is in accordance with the findings of Illert et al. (1982) that Fast Blue labelled less motoneurons in the motor nucleus than HRP.

It is not unlikely that the number of labelled motoneurons demonstrated in this study may represent an underestimate of the real number of such neurons in the MG

motor nucleus. It may arise from the fact that with the retrograde tracing technique, not all motoneurons whose axons are exposed to the same amount of tracer actually take it up. There are numerous factors that can lower the effectiveness of the uptake and retrograde transport of tracers within axonal elements to the parent perikarya (Keizer and Kuypers, 1984; Macchi et al., 1984; Mesulam, 1982). On the other hand, there is a possibility of double-counting of labelled motoneurons at the border of sections. To minimize this error leading to an overestimation, only perikarya with an entire nucleus in the individual sections were taken into account.

Number and size of motoneurons in the motor nucleus of the MG

The distribution of the diameters of the studied motoneurons was bimodal, and the first group of smaller neurons had soma diameters under 27.5 μm; there was no difference in this border value between males and females, and the range of motoneuronal sizes overlapped considerably. Motoneurons with a diameter above the border value were presumed to be alpha, whereas smaller ones were accepted as gamma motoneurons. Hashizume et al. (1988) studied age-related changes in the number and size of motoneurons of the MG motor nucleus in the rat and also found the bimodal distribution of their sizes, with a distinct boundary at about 22.5 μm, which did not depend on the age of animals. Those experiments were performed on male Fischer rats, and the average soma diameter of the motoneuron population ranged from 12.5–45.00 μm, that is, the neurons were smaller than in the present study. A possible explanation of these differences may lie in the subtle differences in the histological method applied, that is, a higher volume of the fixative used by Hashizume et al. (1988). Apart from that, the histochemical procedures employed by those authors and in this study were the same, although not taking into account a correction for shrinkage during fixation. The breed of the rats (Fisher versus Wistar) could also influence the motoneuronal soma size (Fisher rats are smaller). However, it is rather unlikely that the slightly different ways of HRP application, that is, injection into the cut nerve (Hashizume et al., 1988) and a bath of the cut nerve (present study), could account for the larger soma sizes in the latter. A possible reason of the differences could be also the method of diameter measurement (directly under the microscope versus a computer analysis of microphotographs in this study). The bimodal distributions of cell sizes in the MG motor nucleus have also been found in cats and used to classify motoneurons as alpha and gamma ones (Burke et al., 1977). However, the size of motoneurons in female cats was considerably bigger, with the transition between the two subpopulations of cells at 38.5 μm.

The present study revealed that the total number of alpha and gamma motoneurons innervating the studied muscle was significantly higher in males, the difference amounting to 12%. When the motoneurons had been classified as alpha and gamma ones, a bigger sex difference was noted for alpha (a difference of 17% was significant) than for gamma motoneurons (a difference of 4% was not significant).

The main finding that the number of directly stained alpha motoneurons innervating the MG muscle is higher in males than in females is related to the previous results of the motor unit number estimation in that muscle (Celichowski and Drzymała-Celichowska, 2007). Therefore, apart from the evident muscle size differences, there is also a very important sex difference in the motor innervation. The higher number of alpha motoneurons in males (about 17%) seems to be related to the higher mass of their central nervous system (a difference of 17%) noted in the present study.

We have found that the number of gamma neurons in the MG motor nucleus is similar in males and females. A relevant observation has been made by Hashizume et al. (1988), who studied age-related changes in the number of motoneurons in the motor nucleus of the male rat MG. They found that in four age groups (young, middle-aged, old and very old), the number of alpha motoneurons decreased with the age of the animals. The differences in the total number of motoneurons between the younger groups and old and very old animals were considerable (a decrease by 10 and 21% in relation to younger ones, respectively), and the age-related decrease in the number of motoneurons concerned mainly alpha ones (a decrease by 13 and 30% for old and very old groups, respectively), in contrast to the number of gamma motoneurons, which was not significantly reduced in the older groups (a difference of up to 5%). On the other hand, the similar number of gamma motoneurons in males and females may be discussed in relation to other, unknown aspects of muscle physiology. Gamma motoneurons innervate intrafusal muscle fibres in muscle spindles, and therefore, irrespective of the considerable differences in the muscle mass, the present results suggest that muscles in males and females probably have similar number of muscle spindles, but due to their bigger muscle mass, the density of those receptors is lower in males. However, this suggestion must be verified in a separate series of experiments.

An interesting observation concerns the proportion of alpha and gamma motoneurons in the MG motor nuclei of females and males. In females and males, 67 and 70% of motoneurons were alpha, whereas 33 and 30% of gamma motoneurons, respectively. A similar proportion of alpha and gamma motoneurons was also found within the motor nucleus of the MG in female cats (70–75% of

population was presumed to be alpha and 25–30% to be gamma motoneurons) (Burke et al., 1977) and in the male rats (alpha motoneurons ranged from 64% in very old to 72% in young rats, whereas gamma motoneurons ranged from 27% in young to 36% in very old animals) (Hashizume et al., 1988). Apart from above reports, the proportion of gamma and alpha motoneurons in the motor nuclei of other muscles is not known.

Our earlier electrophysiological study of the motor units in the MG muscles of males and females (Celichowski and Drzymała-Celichowska, 2007) allowed us to estimate the number of motor units in this muscle for both sexes based on a comparison between the mean value of motor unit maximum tetanic forces and the muscle force measured during nerve stimulation. The method involved an assumption of linear summation of forces of individual motor units in a muscle. The present results indicate that in using this technique, we have underestimated the number of motor units, determined at 57 for males and 52 for females (a difference of 10%). A possible explanation is the non-linear summation of motor unit forces. It was observed that with the increasing number of co-activated motor units, the effectiveness of summation of their forces evidently decreased (Drzymała-Celichowska et al., 2010). Thus, it seems reasonable that the present study, which offers a direct measurement of the differences in the number of motoneurons in the motor nucleus between males and females, is more precise and reveals that the male muscle contains 17% more motor units than the female one.

The collected data concerning the mass of the rat MG in males and females and the number of alpha motoneurons innervating this muscle offer a possibility to estimate the mean muscle mass innervated by one neuron, in fact, the mean weight of one motor unit. The muscle mass of the rat MG amounts to 1110 mg in males and 660 mg in females (Mierzejewska-Krzyżowska et al., 2011), whereas the mean number of alpha motoneurons determined in this study amounts to 66 and 56, respectively. Therefore, the muscle mass per alpha motoneuron in males amounts to 16.8 mg, whereas in females, this value is substantially lower – 11.7 mg (a difference of 43%). The difference is predominantly due to a higher innervation ratio in males (by about 35% in relation to females) (Mierzejewska-Krzyżowska et al., 2011) and a greater diameter of their muscle fibres (by about 14%) (Mierzejewska-Krzyżowska et al., 2012).

As compared to the number of motoneurons, sex-related differences in the size of gamma and alpha motoneurons were smaller. Males had a significantly greater diameter of gamma motoneurons (9%) and alpha motoneurons (6%) than females. Dimorphic differences in the size of motoneurons were also described by Leslie et al. (1991) for the small foot muscle, flexor digitorum brevis, in the rat: they reported the cross-section area of male motoneurons to be about 10% greater.

The obtained results open some new perspectives for further studies. First, similar experiments should be performed for other muscles (including slow one). Second, the dimorphism of muscle spindles number in skeletal muscles should be studied. Third, a distribution of motoneurons within male and female motor nucleus should be analysed. All these observations will enlarge basic knowledge explaining well-known sex-related differences in motor abilities (Clark et al., 2005; Hakkinen, 1993; Hicks et al., 2001; Kent-Braun et al., 2002; Pincivero et al., 2000, 2003).

In conclusion, the present results revealed significant sex differences in the motor innervation of the rat MG muscle. Male muscles were innervated by about 17% more alpha motoneurons than female ones, whereas the difference in the number of gamma motoneurons was smaller, about 4%. The motoneuronal size differences were smaller, and male motoneurons were about 7% bigger.

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