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ORIGINAL ARTICLE

Gender Differences in the Morphometric Properties of Muscle Fibres and the Innervation Ratio of Motor Units in Rat Medial Gastrocnemius Muscle

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Summary

Previous studies of motor unit contractile properties in the rat medial gastrocnemius revealed that these units generate higher forces in males than in females. Therefore, in the present study the number and morphometric parameters of muscle fibres and the innervation ratio of motor units in the medial gastrocnemius muscle were studied in male and female Wistar rats. The study additionally aimed at determining reasons of gender differences in motor unit force parameters, i.e. the number and diameter of muscle fibres, and mean values of the motor unit innervation ratios. Following staining of reticuline fibres by silver impregnation, the number, diameter and cross-section area of muscle fibres were determined on microscopic images of transverse muscle sections. In males, the muscles were approximately 1.5 times larger by mass and contained about 11 800 muscle fibres, whereas in females the muscles contained around 8000 fibres. In addition, the mean diameter and mean cross-section area of muscle fibres were 14 and 29% larger in males, respectively. Based on previously determined numbers of motoneurons innervating the medial gastrocnemius muscle in male and female rats, the mean innervation ratio, i.e. the number of muscle fibres innervated by one motoneuron, was estimated. This ratio was approximately 26% greater in males compared to females, with values of 207 and 153 fibres per motoneuron, respectively. Therefore, the differences in muscle fibre morphometric parameters and in the innervation ratio are responsible for higher forces of motor units in male muscles.

Introduction

Gender differences are a well-known phenomenon in animals and this dimorphism is evident in the body mass and morphology (Hedrick and Temels, 1989). Some of these differences are connected with diversity in the number and diameter of muscle fibres between the sexes. Muscle fibre number has been analysed in numerous studies without exploring sexual differences. For example, in the medial gastrocnemius muscle of male rats Kanda and Hashizume (1989) found approximately 18 000 muscle fibres, whereas in the same muscle in cats, Burke and Tsairis (1973) found around 170 000 fibres. Gutmann and Hanzlikowa (1966) reported that the rat soleus muscle contained about 2500 fibres. Similarly, several studies have examined the diameter of muscle fibres or skinned muscle fibres. Misawa et al. (2001) reported that the muscle fibre diameter in two fast muscles of the hindlimb of male rat (tibialis anterior and extensor digitorum longus muscles) ranged from 32 to 44 μm and 28 to 44 μm, respectively. For the slow soleus muscle of male rats, the diameter of skinned muscle fibres was found to range from 69 to 111 μm (Alley and Thompson, 1997). However, none of these studies compared these parameters in males and females.

Motor units are composed of one motoneuron and a bundle of muscle fibres innervated by this neuron dispersed within a single muscle, and they represent the smallest functional units participating in motor control processes. According to the classical study of Burke et al. (1973), motor units can be classified into three main physiological types: fast-twitch fatigable (FF), fast-twitch resistant (FR) and slow-twitch (S). Previously, we have examined the contractile properties of various types of motor unit in the medial gastocnemius muscle of male and female rats (Celichowski and Drzymała, 2006). The contraction and the half-relaxation times of motor units were found to be significantly longer in males than in females. On other hand, the forces of motor units were higher in males. Moreover, different proportions of the main types of motor unit were observed, with more FF and less S type units in male muscle.

The force generated by a motor unit correlates strictly with the number of muscle fibres innervated by the motoneuron, i.e. the innervation ratio (Kanda and Hashizume, 1992). The greater force of motor units in male muscles (Celichowski and Drzymała, 2006; Celichowski and Drzymała-Celichowska, 2007) suggests that they are composed of a higher number and/or thicker muscle fibres. Kanda and Hashizume (1992) calculated the innervation ratio for different types of motor unit in the medial gastrocnemius muscle of male rats and found that it ranged from 41 to 80 for S, 116 to 198 for FR, and 221 to 356 for FF type units. In the slow soleus muscle of rats, all motor units are of the S type and they contained from 58 to 84 muscle fibres, whereas the innervation ratio of the fast tibialis anterior muscle in male rats ranged from 46 to 57 for FR and 127 to 178 for FF type motor units (Larsson and Ansved, 1995).

Previously, we calculated the number of motor units in the medial gastrocnemius muscle in male and female rats using an electrophysiological method based on a comparison of the force generated by the muscle to the mean force of individual motor units (Celichowski and Drzymała-Celichowska, 2007). This study revealed that the number of motor units in the medial gastrocnemius muscle is different in the two sexes: in males the muscle was composed of approximately 10% more motor units than in females (57 and 52 motor units, respectively). Using the known differences in the proportional composition of motor units in the medial gastrocnemius muscle it was possible to determine that this muscle is composed of approximately 26 FF, 23 FR and 8 S units in males, and 21 FF, 19 FR and 12 S motor units in females. It should be stressed that the mass of this muscle differs considerably (by approximately 40%) between the sexes, with mean masses of 1 030 and 750 mg for males and females, respectively (Celichowski and Drzymała-Celichowska, 2007). As mentioned above, the sexual difference in the number of motor units in the medial gastrocnemius muscle is much smaller (approximately 10%). These findings suggest that the innervation ratio for the gastrocnemius muscle is higher in males than in females. The same hypothesis is supported by higher forces of motor units in male muscles (Celichowski and Drzymała, 2006; Celichowski and Drzymała-Celichowska, 2007). On other hand, the ability of male motor units to generate higher forces can also be an effect of differences in muscle fibre diameters. Study of muscle fibres of vastus lateralis muscle of young men and women revealed that the cross-section areas of the three major fibre types (I, IIA, IIB) were larger for man (Staron et al., 2000).

The aim of this study was to demonstrate sexual differences in the morphology of the medial gastrocnemius muscle in rats, especially in the number and diameter of muscle fibres, and also to estimate mean values of the motor unit innervation ratios. These parameters of skeletal muscles are basic data for understanding the differences in motor control processes between males and females.

Materials and Methods

The experiments were performed on five 6-month-old male Wistar rats (mass 400–490 g) and five 6-month-old female Wistar rats (mass 245–290 g). The animals were maintained in cages at standard conditions with a constant temperature of 20 ± 1°C and a 12 h light–dark cycle and were fed *ad libitum* a complete laboratory diet 'Labofeed B' (Poland). All procedures were approved by the Local Ethics Committee and followed European Union guidelines on animal care as well as Polish Law on The Protection of Animals. Rats were deeply anesthetized with sodium pentobarbital (60 mg/kg, i.p., initial dose, supplemented as required). At the end of the experiment the animals were euthanized by an overdose of sodium pentobarbital (180 mg/kg i.p.).

In rats of both genders, the medial head of the gastrocnemius muscle of the right and left hindlimbs was exposed, isolated from surrounding tissues and excised. After weighing, the muscles were fixed in 10% formalin solution for 3–4 weeks and embedded in paraffin. The muscles were then cut transversely using a microtome into 5 μm serial sections. Five sections, taken from the middle part of the muscle, where the cross-section was largest, were mounted on chrom-alum-gelatin-coated glass slides, air-dried overnight at room temperature and stained by silver impregnation of reticuline fibres to demonstrate the morphology of muscle fibres (Beech and Davenport, 1933; Brooke et al., 1971; Burck, 1973; Puchtler and Waldrop, 1978; Bradbury and Gordon, 1980; Sheehan and Hrapchak, 1980).

All mounted sections were examined using a Jenaval microscope (Carl Zeiss, Jena, Germany) (magnitudes:

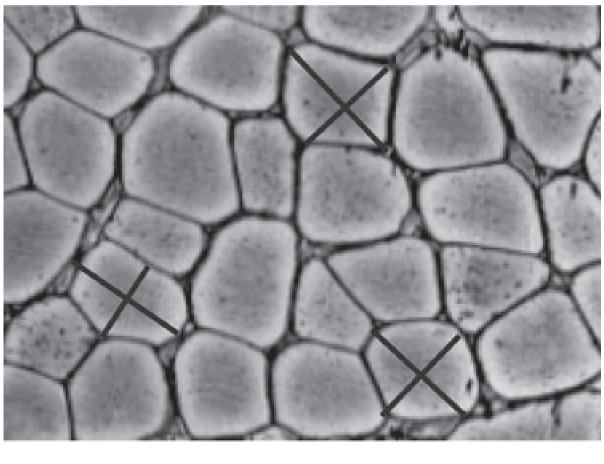

Fig. 1. Cross-section of the rat medial gastrocnemius muscle stained by silver impregnation of reticuline fibres technique showing the method used for the determination of muscle fibre diameter. The *long lines* represent the maximum diameters of the muscle fibres, whereas the *shorter perpendicular lines* are the diameters used in the analysis. The *scale bar* is 67 μm.

1 ×, 12.5 ×, 40 × and 100 ×), recorded using a Sony CCD-Iris Camera and the digitized images were transferred to the MultiScanBase System for Windows (Computer Scanning System, version 14.02, Warsaw, Poland).

One section from each muscle was taken for measurements. First, the muscle transverse cross-section area was calculated. The high-power image of the section was photographed in fragments, then these fragments were printed and a magnified image of the muscle cross-section was reconstructed. Using this image, the number of muscle fibres present in the cross-section was calculated. Ten random microscope pictures (under magnification 40 ×) taken from deep and superficial parts of the cross-section were then recorded, and for approximately 100 muscle fibres visible on each of these images, the area of single fibres (μm^2) and the fibre diameters (μm) were determined. According to the criteria of Dubowitz (1985), the lesser diameter of the muscle fibre was used in calculations (Fig. 1).

Statistical analysis was performed using the STATISTICA program (StatSoft, version 8.0, Cracow, Poland).

For statistical evaluation of significances of differences between the properties of male and female muscles the Mann–Whitney *U*-test was used.

Results

Examples of microscopic images of cross-sections of the medial gastrocnemius muscle from male (a) and female (b) rats are shown in Fig. 2.

Table 1 presents the mean values, standard deviations and variability ranges for body mass, muscle mass, ratio of muscle mass to body mass and also the cross-section area of the medial gastrocnemius muscle for male and female rats. In male rats, the body and the muscle masses were significantly higher (Table 1), and the cross-section area of the muscle was larger than in females (Table 1). The ratio of muscle mass to body mass was higher for females, although the difference between the sexes was not significant (Table 1).

The mean values, standard deviations and variability ranges for the number, diameter and cross-section area of muscle fibres in the medial gastrocnemius muscle for male and female rats are presented in Table 2. The mean number of muscle fibres within the studied muscle was approximately 47% higher in males than in females (Table 2). The mean diameter of muscle fibres also differed significantly and was 14% greater in males (Table 2). The mean cross-section area of muscle fibres was 29% larger in males, and also the difference between the sexes was significant (Table 2).

The innervation ratio of motor units in the medial gastrocnemius muscle, calculated by dividing the mean values of the number of fibres in the studied muscle for male and female rats (11 772 and 7 931 fibres, respectively) by our previously published estimates of the number of motor units in this muscle (57 and 52 units, respectively) (Celichowski and Drzymała-Celichowska, 2007) showed a clear gender difference:

Males: $11\,772/57 = 206.53 \approx 207$ muscle fibres innervated by one motoneuron;

Females: $7\,931/52 = 152.52 \approx 153$ muscle fibres innervated by one motoneuron.

Discussion

Analysis of the morphometric parameters of the medial gastrocnemius muscle and its fibres in rats of both genders revealed significant sexual differences in the cross-section area of the muscle and the total number of muscle fibres, with a relatively smaller difference in the diameter of the fibres. These three muscle parameters were approximately 71, 47 and 14% larger in males than in females, respectively. Therefore, the main factor responsible for the greater force generation ability of males compared to females is the higher number of fibres in their muscles. Previously, we compared the force generated by the medial gastrocnemius muscle during stimulation of its nerve with the mean force of isolated motor units and found that this muscle was innervated by approximately 10% more motoneurons in male rats than in females (Celichowski and Drzymała-Celichowska, 2007). This relatively small difference in the number of motoneurons

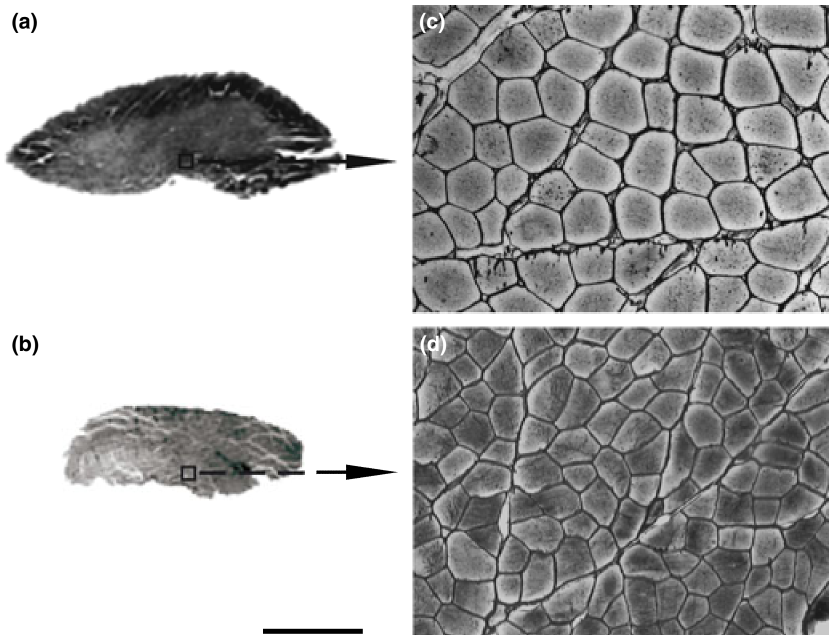

Fig. 2. Examples of microscopic images of cross-sections of the medial gastrocnemius muscle from male (a) and female (b) rats; dark squares illustrate areas of magnified fragments of the middle part of these cross-sections for the male (c) and female (d). The *scale bar* represents 4 mm for (a) and (b), and 126 μm for (c) and (d).

Table 1. Mean values, variability ranges and standard deviations of the body mass, muscle mass, ratio of muscle mass to body mass and the cross-section area of the muscle of the medial gastrocnemius of male and female rats. The statistical significance of differences between males and females (the Mann–Whitney *U*-test) is indicated under the table: ** difference significant at $P < 0.01$, * difference significant at $P < 0.05$, n.s. difference not significant, $P > 0.05$.

Gender	body mass [g] **	muscle mass [g] **	muscle mass/ body mass [%] n.s.	cross-section area of the muscle [mm^2] *
Male (n = 10)	445 ± 37	1.11 ± 0.15	0.25 ± 0.02	32.4 ± 6.4
range	400–490	0.94–1.35	0.23–0.28	19.7–44.9
Female (n = 10)	248 ± 5	0.66 ± 0.07	0.27 ± 0.03	19.0 ± 2.3
range	240–255	0.56–0.81	0.23–0.33	15.4–22.4

Table 2. Mean values, variability ranges and standard deviations of the total number of muscle fibres, fibre diameter and the cross-section area of muscle fibres of the medial gastrocnemius of male and female rats. The statistical significance of differences between males and females (the Mann–Whitney *U*-test) is indicated under the table: ** difference significant at $P < 0.01$, * difference significant at $P < 0.05$.

Sex	total number of muscle fibres **	muscle fibre diameter [μm] **	cross-section area of muscle fibres [μm^2] *
Male (n = 10)	11 636 ± 1 558	57.99 ± 6.41	2 943 ± 585
range	7 479–14 373	30.66–88.00	1 297–5 810
Female (n = 10)	7 913 ± 1 570	50.86 ± 4.89	2 292 ± 591
range	5 199–9 599	35.73–72.46	1 065–4 572

innervating this muscle in the two genders and the much larger difference in the total number of muscle fibres in males and females found in the present study indicated that male muscles have higher innervation ratios than those of females. The mean innervation ratio calculated for the medial gastrocnemius muscle was 35% higher in males than in females, with values of 207 and 153, respectively. It should be stressed that until now, sexual differences in the properties of motoneurons, including morphometric and electrophysiological parameters, the axonal conduction velocity and innervation ratios, had not been investigated.

The innervation ratio has been studied for several muscles of the cat and rat. Using a glycogen depletion technique, Burke and Tsairis (1973) found mean innervation ratios of 380 for FF and 200 for FR units in the cat medial gastrocnemius muscle. Chamberlain and Lewis (1989) studied rat soleus muscle, composed exclusively of slow motor units, and found that the innervation ratio was lower (110 fibres per unit). Kanda and Hashizume (1992) studied motor units in the rat medial gastrocnemius muscle and found that the innervation ratio ranged from 62 to 356 muscle fibres per motoneuron. Moreover, they determined that the ratio ranged from 62 to 80 for S motor units, 116 to 198 for FR units and 220 to 356 for FF units. The proportions of the three types of motor unit in the rat medial gastrocnemius muscle was determined by Kanda and Hashizume (1992) for males, as well as by Celichowski and Drzymała (2006) for males and females. Kanda and Hashizume (1992) found that this muscle was composed of 83% fast (FF and FR) units and 17% slow units. We found similar results for males (86% fast and 14% slow units), but a lower proportion of fast units in females (74% fast and 26% slow units) (Celichowski and Drzymała, 2006). In the present study, the estimated mean innervation ratio in male rat medial gastrocnemius muscle (composed predominantly of fast motor units) was 207 fibres per motoneuron, which falls between the value ranges for FF and FR units determined by Kanda and Hashizume (1992). Interestingly, the difference between the innervation ratios for the two genders was similar to the difference in the forces of their motor units reported previously. In male rats, the mean maximum tetanic force of motor units in the medial gastrocnemius was 144.3 mN, whereas for females it was 100.8 N, i.e. a difference of 43% (Celichowski and Drzymała-Celichowska, 2007), while the difference in innervation ratio was 35%.

The calculation of mean innervation ratios of motor units in male and female muscles was based on previously estimated number of motor units (Celichowski and Drzymała-Celichowska, 2007) – this estimation was performed by comparison of the mean motor unit maximum tetanic force to the force of a muscle measured during the nerve stimulation (Gardiner and Olha, 1987; Einsiedel and Luff, 1992). However, this method can be imprecise because the forces of individual motor units in the studied muscle summate less-than-linearly, as shown in a recent paper (Drzymała-Celichowska et al. 2010). Under such circumstances the maximum tetanic force during the nerve stimulation is generated by the contraction of more motor units than results from a simple algebraic calculation. As a consequence, the number of motor units calculated for male and female medial gastrocnemius muscles (Celichowski and Drzymała-Celichowska, 2007) is probably underestimated. One may expect that a possible error is similar for both genders. Nevertheless, if the real number of motor units is higher, the innervation ratios are lower than calculated in this paper. Due to a lack of detailed and unequivocal data concerning the mechanisms of force summation it is impossible to estimate the absolute value of this error in the calculations of the innervation ratio.

The differences of the motor unit forces between male and female muscles were noted for all three types of motor units, and ranged between 11 and 49%. For the male rat medial gastrocnemius the maximum tetanic forces were higher and amounted to 48, 109, and 206 mN for S, FR, and FF types, respectively, whereas for female muscle the maximum values of this parameter were 43, 73 and 158 mN for S, FR and FF units, respectively (Celichowski and Drzymała-Celichowska, 2007). As shown by Kanda and Hashizume (1992), the motor unit force strictly correlates to a number of muscle fibres within a unit (the innervation ratio). Therefore, one may suppose that the innervation ratios for the three motor unit types are higher in male muscles.

The direct measurement of the number of muscle fibres within the studied muscle and the calculation of innervation ratios permit the approximation of the force generated by individual muscle fibres. Our previous measurements of the tetanic forces of male and female medial gastrocnemius muscles gave maximum values of 8260 and 5250 mN, respectively (Celichowski and Drzymała-Celichowska, 2007). Taking into account the mean number of muscle fibres in the rat medial gastrocnemius (11 772 in males and 7931 in females), the maximum force generated by one fibre in the male muscle comes to approximately 0.701 mN, whereas in the female muscle this value is slightly lower at 0.661 mN. This difference may be due to the smaller diameter of muscle fibres in the female muscle identified in this study. Furthermore, the available data concerning the mean tetanic force of the three types of motor units in male medial gastrocnemius (48 mN for S, 109 mN for FR and 206 mN for FF type motor units) (Celichowski and Drzymała-Celichowska, 2007), together with the mean values of the innervation ratios for these motor unit types (66 fibres for S, 154 for FR and 271 for FF type motor units) (Kanda and Hashizume, 1992) permit an approximation of the mean force generated by one muscle fibre: 0.727, 0.707 and 0.760 mN for fibres of the S, FR and FF motor unit types, respectively. Unfortunately, the data required to perform comparable calculations for muscle fibres within female motor units are not available.

The gender differences in the innervation ratios of the three types of motor units suggest differences in the mean size of their motoneurons. Ulfhake and Kellerth (1982) found significant differences between the cell bodies and first-order dendrites of α-motoneurons supplying different types of motor unit in the cat gastocnemius and soleus muscles. Slow motoneurons (S) and fast motoneurons

(FF) in this muscle were compared, and slow motoneurons had a smaller soma diameter (58.2 μm for S and 62.6 μm for FF) and smaller soma surface area (10 160 μm^2 for S and 11 290 μm^2 for FF). In addition, FF and FR motoneurons had proportionally larger dendritic trees than the S units. Jami and Petit (1975) studied four muscles of the cat hindlimb and found a significant correlation between motor unit tetanic tension and axonal conduction velocity. Moreover, a similar significant correlation between the maximal tetanic force and axonal conduction velocity, was described in fatigue-resistant units (S and FR), although this correlation was weak or absent in the FF unit group (Emonet-Dénand et al., 1988). Even in early studies of muscle innervation it was assumed that there is a correlation between axonal size and the number of muscle fibres innervated (Eccles and Sherrington, 1930). Ulfhake and Kellerth (1982) suggested that the number of dichotomous divisions of a motor axon depends on the axonal diameter, so the number of muscle fibres innervated is related to axon size.

In conclusion, the main factor responsible for gender differences in muscle mass in rats is the number of muscle fibres: in the medial gastrocnemius muscle, males have around 47% more fibres than females. In addition, the cross-section area of male muscle fibres is about 29% larger. Lastly, the innervation ratios for motor units in male muscles are approximately 35% higher than those of females. Therefore, the two differences are responsible for higher forces of motor units in male muscles.

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