CHAPTER 4

roximo-distal organization and fibre type regionalization in rat hindlimb muscles

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Abstract

Five muscles of the rat's lower hindlimb were compared with regard to their histochemical fibre type distribution at seven different proximo-distal levels. The muscles were: extensor digitorum longus (ED), flexor digitorum and hallucis longus (FD), gastrocnemius medialis (GM), peroneus longus (PE) and tibialis anterior (TA). In all the five muscles, the relative density of the “slow” type I fibres showed a striking and similar decrease from proximal toward more distal levels. In addition, the type I fibres were concentrated within smaller and more eccentrically placed regions at distal than at more proximal levels. As a background for the further analysis of these lengthwise aspects of type I fibre regionalization, architectural features of the muscles were determined. Pinnation angles and the position of major tendons and tendon sheets were assessed in fresh specimens. Muscle fibre lengths were measured for single fibres dissected from different regions of macerated muscles. In all cases, fibre length was much shorter than muscle length (mean fraction ranging from 21 to 55%), implying that the proximo-distal changes in histochemical fibre properties were indeed explainable as being due to gradual lengthwise changes in fibre type populations. The similarity of these lengthwise changes across the muscles was in contrast to their differences in other aspects of functional organization, such as the average density of type I fibres and architectural features determining their relative capacities for shortening and force generation. The possible functional role of the proximal accumulation of type I fibres was discussed in relation to circulation and thermal balance; besides, the proximo-distal regionalization of type I fibres might (partly) reflect processes that had been associated with early stages of muscle differentiation. Furthermore, the results underline that, when determining the fibre type composition of rat hindlimb muscles, identifying the proximo-distal level of sampling is a matter of great importance.

Key words: Myosin ATPase; Slow muscle fibres; Fiber type differentiation; Hindlimb organization; Muscle architecture

Introduction

In the control of motor behaviour, most muscles have to be used for rapid movements as well as for the prolonged maintenance of posture. Such different types of motor activity are associated with different requirements for the power, speed and endurance of the skeletal muscles. Correspondingly, muscles contain fibres with widely varying contractile and biochemical properties, optimally suited for different kinds of motor function. A major subdivision concerns that between “fast” and “slow” fibres, and a coarse categorization along these lines can be made using standard histochemical methods (myofibrillar ATPase). The present study concerns the lengthwise organization of slow fibres in hindlimb muscles of the rat.
The fibre type composition of skeletal muscles has typically been studied in cross-sections taken from the “muscle belly”, i.e. from the thickest portion, often located somewhere around the proximo-distal middle of the muscle. Such investigations have shown that muscles differ considerably in their fibre type composition (e.g. Ariano et al., 1973; Armstrong and Phelps, 1984) and that, within midlevel cross-sections, there are also large and systematic regional differences in the relative densities of slow and fast muscle fibres (“fibre type regionalization”, review: Kernell, 1998). Lengthwise differences in fibre type composition have also occasionally been described in various animals (e.g. English and Letbetter, 1982; Donselaar et al., 1987; Gardiner et al., 1991; Totland and Kryvi, 1991; Lexell et al., 1994; Brandstetter et al., 1997; Punk et al., 1998), but few extensive studies have been made. This is an important problem because in many muscles different fibres may indeed be seen within cross-sections taken at different proximo-distal levels. This is true for all pinnate muscles and there are also examples known of parallel fibre arrangements in which different fibres are serially linked (e.g. Loeb et al., 1987; Heron and Richmond, 1993). Single muscle fibres are generally assumed to have a largely homogeneous composition along their length. However, muscle fibres are multi-nucleate cells which might even, potentially, express different contractile proteins at different sites along their length; fibres with such properties have been reported for the frog’s tibialis anterior (Edman et al., 1985; Edman et al., 1988) and, during development, for the rat’s soleus (Sakuma et al., 1995).

In the present paper, five different muscles of the rat’s lower hindlimb were compared with regard to their proximo-distal distribution of slow fibres and their general architectural features. Striking proximo-distal gradients of type I fibre density and regionalization were found in all investigated cases. Some of the present findings have been presented in a congress abstract (Wang and Kernell, 1998).

Methods

The measurements were made on muscles from adult female Wistar rats, weighing 220-295 g. Before the operations, the animals were kept in conventional plastic cages and fed *ad libitum* with standard laboratory food and tap water.

General preparative procedures

Prior to the dissection of the muscles, the animals were anaesthetized with pentobarbitone (50 mg/kg i.p.). We studied the following five muscles: extensor digitorum longus (ED), flexor digitorum and hallucis longus (FD), gastrocnemius medialis (GM), peroneus longus (PE) and tibialis anterior (TA). Flexor digitorum and hallucis longus was treated as one muscle; in our cases, these two muscles seemed fused and could not be separated without causing tissue damage. In all cases except those concerning measurements of muscle fibre length (see below), the observations were restricted to one limb in each rat (left side).

With reference to Greene’s “Anatomy of the Rat” (1935), each one of the studied muscles was gently exposed and dissected free from the surroundings.
The length of each muscle was measured from the most proximal appearance to the ultimate distal disappearance of muscle tissue; the knee was then kept fully extended and the ankle at 90°. Before cutting the tendons, the posterior and/or lateral sides of each muscle were marked with water-insoluble coloured stains (yellow and blue), using a fine painter’s brush. Tendons were cut and the muscle was fixed to a metal clamp at its measured in situ length, covered with talcum powder and fixed by immersion in isopentane kept at its freezing point by liquid nitrogen. Thereafter, muscles were stored in a freezing box at -80°C until further processing.

After the end of the dissections, the animals were killed with an overdose of pentobarbitone (i.p.)

Sectioning and staining procedures

Muscles from eight rats were processed for histochemical measurements. Using a cryostat (kept at -20°C), a set of 16 serial sections of 10 µm were cut at each of seven equally spaced proximo-distal levels for each muscle (Fig. 1). These “analysis levels” were spaced such that about 0.5 inter-level length of muscle was still present above the first and below the seventh level. Considering the lengths of these muscles (Table 4), the inter-level distances varied between about 3 and 4.5 mm. Level 4 was identical to the “midlevel” analyzed by Wang and Kernell (2000).

The serial sections obtained from each proximo-distal level were stained for myofibrillar ATPase using standard techniques (mATPase; Lind and Kernell, 1991).

General procedures for the measurement of type I fibre density and regionalization

High-contrast sections stained for mATPase after acid preincubation were used, showing “slow” type I fibres as black against a weakly stained background. The anatomical measurements were made on high-contrast photocopies of such muscle cross-sections, using a graphic tablet connected to a PC with custom-made software. The photocopy was made directly from the stained histological section using a micro-fiche copying machine with exchangeable lenses (Canon PC Printer 70M). All ambiguous details on the photocopy were evaluated by examining the original section through a microscope at high magnification. The graphic tablet was used for tracing the outline of the muscle cross-section and any substantial “empty” pieces inside the muscle (e.g. tendon sheets). Furthermore, across the whole muscle section, the position of each type I muscle fibre was digitized and stored in a computer file. On the graphic tablet, muscle images were routinely positioned with the posterior muscle side at the top and lateral toward the left (cf. Figure 1).

Traced data were stored in computer files for further analysis. Custom-made software calculated the cross-sectional area of the muscle (MArea; any substantial “empty” regions not included). As a linear measure of muscle size, the equivalent muscle diameter (EqD) was calculated from MArea using the formula for a circle. The overall density of the type I fibres (FibD) was expressed as the number of fibres/mm². Furthermore, after determining the positions of all the type I fibres, the program made calculations...
resulting in several measures for fibre type regionalization.

Measures of fibre type regionalization

Figure 1 shows examples of how the type I fibres were distributed within muscle cross-sections. As we have recently analyzed for midlevel sections (Wang and Kernell, 2000), there were two ways in which the fibre type regionalization expressed itself:

*Direction and degree of type I fibre eccentricity: “vector regionalization”.*

In most muscle cross-sections, the population of type I fibres was clearly eccentrically located. This was often evident also in cases for which the type I fibre region covered most of the cross-section. We quantified this aspect of regionalization by computing a “type I fibre vector”, extending from the centre of the cross-section to the centre of the type I fibre cluster (arrows, Figure 1). The geometrical centre of the cross-section was found by calculating the centre of mass of a sheet with the same shape and a uniform thickness and density. Correspondingly, the geometrical centre of the type I fibre population was found by computing the centre of mass for all the type I fibre positions, each fibre site being represented by a point with an equal amount of mass. The measured inter-fibre distances were used for applying standard rules of moment-arms in recursive calculations. One advantage of this procedure is that a position of the population-centre can be unequivocally computed for fibre distributions of any shape, skewedness, size or extent.

We expressed the length of the type I fibre vector (VL) as a percentage of the equivalent muscle diameter (EqD, see above). The direction of the vector, the “vector angle” (VA), was given in degrees and with directions ordered counterclockwise according to standard rules of trigonometry. The vector arrow was pointing medially for 0° and 360°, posteriorly for 90°, laterally for 180° and anteriorly for 270°.
Size of type I fibre region: “area-regionalization”.

Muscle cross-sections differed with regard to the relative size of the region containing the type I fibres, as compared to the total cross-section area (MArea). In Figure 1 this “type I fibre region” (FR) was, for instance, relatively large for proximal levels and smaller for distal levels. We quantified this aspect of fibre type regionalization by calculating the size of the type I fibre region, expressed as a percentage of the whole cross-section area. In doing these calculations we used a “sector method” for approximately delineating the type I fibre region (FRs). In this method, the space surrounding the “middle” of the fibre population (i.e. its calculated “centre of mass”) was subdivided into several equal-angle sectors and, within each sector, the fibre most remote from the cluster centre was identified. The outer border of the type I fibre region was then defined by joining these most remote fibres with straight lines (Figure 1, interrupted lines). Due to the manner in which these lines are calculated, occasional fibres may be “lost” and fall just outside the circumscribed area (cf. Figure 1, level 6). However, provided the number of sectors is adequate, the “lost” fibres will typically be very close to the regional border and such inaccuracies will not produce very complex and “unnatural” looking cluster-borders (for further discussions of this and alternative methods, see Kernell and Wang, 2000).

Measurements of fibre cross-section areas and calculations of type I fibre frequencies

In two of the present muscle species (four ED muscles; four GM muscles) measurements of muscle fibre cross-section area were made within sections from proximo-distal levels 2, 4 and 6 (cf. Figure 1). These measurements were typically done in sections stained for mATPase after acid preincubation. A distinction was made between type I (dark) and type II fibres (moderate - light) and between fibres sampled from within the “type I fibre region” and from outside this region. On average about 190 fibres were measured within each section, with the sampling distributed as widely as possible across the relevant region. The measurements were performed using a microscope at high magnification, connected to a video-camera and a PC. Using a video-display and commercial software (Bioscan Optimas), fibre outlines were traced with a mouse and calculations were made of fibre cross-section area.

For single cross-sections, the overall percentage of type I fibres was calculated from the following measurements: (1) the cross-sectional areas of type I and II fibres (Fa1 for type I; Fa2i and Fa2o for type II from within and outside the type I region); (2) the total number of type I fibres (N1); (3) the muscle cross-sectional area (MArea); (4) the area of the type I fibre region (FRs, here given in µm²). In doing these calculations the assumption...
was made that muscle fibres accounted for about 85% of the total muscle volume (Gollnick et al., 1981). This assumption implies that the summed cross-sectional area of the muscle fibres would relate to that of other components as \((85 / 15)^{2/3}\), which gives a summed fibre area equal to about 76.1% of the total cross-sectional area \((Cf)\). The total area occupied by type I fibres equalled:

\[
A1 = N1 \times Fa1
\]

The total area occupied by type II fibres within the type I fibre region was calculated as

\[
A2i = (Cf \times FRs) - A1
\]

and the number of these type II fibres equalled

\[
N2i = A2i / Fa2i
\]

Similarly, the number of type II fibres lying outside the type I fibre region was calculated as

\[
N2o = (Cf \times (MArea - FRs)) / Fa2o
\]

Finally, the overall percentage of type I fibres was calculated as

\[
%I = 100 \times (N1 / (N1 + N2i + N2o))
\]

Measurements of muscle fibre length and muscle architecture

Pinnation angles and major tendon arrangements.

These determinations were made on freshly dissected muscles from three rats. Muscle lengths and pinnation angles were measured in muscles lying on graph-paper. Prior to the measurements, the muscle was stretched and allowed to shorten passively (i.e., there was no slack). Pinnation angles were measured in relation to the lengthwise axis of the whole muscle (i.e., not in relation to the direction of local tendon sheets) and with the muscle rotated such that these angles were maximized. Under the same conditions, we also made determinations of approximate fibre lengths. In the fresh preparation, the ends of the fibres were difficult to ascertain and these measurements typically gave shorter values than those obtained for the dissected fibres. For the further calculations, we exclusively used the length values obtained from the dissected fibres.

Length of dissected muscle fibres.

Muscles from five limbs of three rats were used for these determinations. In each limb, the five studied species of muscle were dissected free from the surroundings (ED, FD, GM, PE, TA). Muscle length was measured with the knee stretched and the ankle at right angle, and the posterior and/or lateral side of each muscle was labelled with water-insoluble dye. After being removed from the limb, each muscle was fixed by strings to a piece of wood at its \textit{in situ} length. It was then immersed in HNO\textsubscript{3} for maceration, using methods similar to those of Gollnick et al (1981). Generally, treatment with a series of increasing concentrations of HNO\textsubscript{3} (0.5 to 15 %) during totally about 10-12 h produced a degree of muscle maceration sufficient for the dissection of single muscle fibres. The length of single fibres was measured with a pair of calipers, using a dissection microscope. In each muscle, 20 dissected fibres were sampled.
from each one of four regions: (i) “proxi-
mal-I and II “, from the upper third of the
muscle and from the side at which the type
I fibres were typically accumulated (i.e.
from the side indicated by the type I fibre
vector for the same muscle species and
proximo-distal level); (ii) “proximal-II”,
as (i) but from the opposite side of the
muscle; (iii) and (iv) as (i) and (ii) but
from the distal third of the muscle.

Using the present measurements of
muscle length (Mlen), fibre length
(FibLen) and fibre pinnation angle
(PenAng), we calculated the “physiologi-
ical cross-section area” (CSA) in a way
similar to that of Wickiewicz et al. (1983),
i.e.

\[
CSA = \frac{\cos(PenAng) \times Mwt}{FibLen \times Md}
\]

where Mwt is muscle weight and Md the
density of muscle tissue. Values for
muscle weight were averages of measure-
ments recently obtained for the present
target muscles in rats of the same strain,
sex and weight-range (Wang and Kernell,
2000). The density of muscle tissue was
set to 1.072 g / ml (Gollnick et al., 1981).

For the same muscle species and
sampling site, mean fibre lengths from the
left and right sides of the same rat were
commonly as different as those from dif-
ferent rats. Therefore, we chose to ana-
lyze these data with each limb treated as
a separate “sampling entity” (see Table
3).

Statistics

Whenever applicable, mean values are
given ± SD. Pearson correlation coeffi-
cients were calculated for analyzing the
degree of co-variation between different
variables. Differences in properties be-
tween different groups of muscles were
analyzed using standard t test procedures.
Differences between samples of single
dissected muscle fibres were analyzed
using ANOVA procedures. Calculations
were made using Excel (Microsoft) and
the software package SYSTAT. Cases
with P < 0.05 were considered statistically
significant.

Results

Number and density of type I fibres at dif-
ferent proximo-distal levels

We measured the density of type I fibres
as the number of fibres per unit cross-sec-
tion area. Figures 2A and B show how
the two basic parameters involved, the
cross-sectional muscle area and the total
number of cross-sectioned type I fibres,
varied with proximo-distal level in the
five studied muscles. The various muscles
clearly had very different shapes: GM and
TA exhibited marked and protruding
muscle “bellies” whereas PE was almost
equally thick at all levels. If the density
of type I fibres were the same at all
proximo-distal levels, then the total num-
ber of type I fibres would be distributed
in a manner similar to that for the cross-
section areas. Superficially, Figure 2B
might look as if this were the case for sev-
eral of the muscles: a marked peak ap-
pears for GM and less pronounced level-
related maxima are shown for ED, FD and
TA. However, already this superficial
scrutiny reveals a marked difference for
one of the muscles: in PE the number of
type I fibres declines monotonically from
the most proximal section and down. Cal-

Table 1. Correlations between proximodistal level and type I fibre distribution

<table>
<thead>
<tr>
<th>Muscle</th>
<th>FibD</th>
<th>FRs</th>
<th>VL</th>
<th>VA</th>
</tr>
</thead>
<tbody>
<tr>
<td>ED</td>
<td>-0.98</td>
<td>-0.96</td>
<td>+0.97</td>
<td>+0.81</td>
</tr>
<tr>
<td>FD</td>
<td>-0.97</td>
<td>-0.93</td>
<td>+0.95</td>
<td>ns</td>
</tr>
<tr>
<td>GM</td>
<td>-0.97</td>
<td>-0.95</td>
<td>ns</td>
<td>-0.96</td>
</tr>
<tr>
<td>PE</td>
<td>-0.99</td>
<td>-0.96</td>
<td>+0.97</td>
<td>(+0.73)</td>
</tr>
<tr>
<td>TA</td>
<td>-0.94</td>
<td>-0.93</td>
<td>+0.89</td>
<td>(-0.69)</td>
</tr>
</tbody>
</table>

Correlation coefficients calculated for average values of each parameter vs. proximodistal level (n = 7). Abbreviations: FibD, density of type I fibres; FRs, area of type I fibre region (% of muscle cross-section area), calculated using the “sector method”; VL, relative length of the type I fibre vector (% of equivalent muscle diameter); VA, angle of type I fibre vector; ns, P > 0.1. For values in paranthesis, 0.1 > P > 0.05. For all other values, P < 0.05 or better. Muscle abbreviations: ED, extensor digitorum longus; FD, flexor digitorum and hallucis longus; GM, gastrocnemius medialis; PE, peroneus longus; TA, tibialis anterior.

Fig. 2. Changes in muscle size and composition with proximo-distal level. Each data point is an average (± SE for A-C) for measurements from six to eight rats, as obtained from cross-sections taken at the indicated proximo-distal level (cf. Figure 1). Different symbols for each one of the five investigated muscles (abbreviations as in Table 1). (A) Total cross-section area (mm²). (B) Number of type I fibres. (C) Type I fibre density (number of type I fibres/mm² cross-section area). (D) Normalized values for type I fibre density (%; means of panel C normalized vs. the maximum mean value for each muscle).
Calculations of the actual type I fibre density for each proximo-distal level reveals that, in this respect, all the studied muscles showed a similar behaviour. Although their absolute values for type I fibre density varied considerably (Figure 2C) they all showed an almost identical decline of the relative type I fibre density from proximal to distal levels (Figure 2D; see also Figure 1). This tendency was statistically significant for each one of the five muscles (Table 1). In several cases, the most distal section did not contain hardly any type I fibres at all (1 or 0 fibres); this was true for most of the GM and for about half of the FD and TA muscles.

**Fibre diameters at different proximo-distal levels**

In the analysis of Figure 2, the proximo-distal distribution of type I fibres was expressed as the number of type I fibres/mm² (“density”) rather than by the more commonly used indication of fibre type representation (relative “frequency” of type I fibres, %). For a given region, the relationship between the type I fibre density and frequency depends on the numbers and mean cross-sectional areas of the main fibre types. Table 2 shows average data for the cross-sectional fibre areas in a subset of ED and GM muscles. As compared with the level-related differences in fibre cross-sectional areas (Table 2), the proximo-distal variations in type I fibre density were very large (Figure 2D). Hence, as one might intuitively expect, there was in both muscle species a strong and similar correlation between the type I fibre density (fibres/mm²) and the calculated type I fibre frequency (%) (Figure 3A). The proximodistal decline of type I fibre representation was as evident for the frequency of type I fibres (%) as for type I fibre density (cf. Figure 2C and

| Table 2. Mean fibre areas at different proximo-distal levels |
|----------------|----------------|----------------|
|                | Level 2        | Level 4        | Level 6          |
|                | 997±40         | 1277±69        | 1039±133         |
| Type I         | 1377±105       | 1909±122       | 1676±289         |
| Type IIi       | 1890±361       | 2601±154       | 2501±564         |
| Type IIo       | 1449±146       | 1379±174       | 1254±156         |
| Type I         | 1562±93        | 1610±132       | 1822±224         |
| Type IIo       | 2064±215       | 2683±236       | 3145±237         |

Means ± SD for cross-sectional areas (µm²) of muscle fibres of histochemical types I and II, as measured at proximo-distal levels 2 (proximal), 4 (midlevel) and 6 (distal). For type II fibres, a distinction is made between fibres found within (IIi) or outside (IIo) the type I fibre region. Each average is the mean of values from four different rats. On average about 190 fibres were measured for each level and individual muscle. For each line, the statistical significance of differences between neighbouring mean values is indicated (paired t tests, * P<0.05 or better; + 0.1 > P > 0.05; ns P > 0.1). Last column: comparisons of Level 2 vs. Level 6.
LENGTHWISE MUSCLE ORGANIZATION

Fig. 3. (A) Relative frequency of type I fibres (%) plotted vs. their density (fibres/mm²) for extensor digitorum longus (ED, filled triangles) and gastrocnemius medialis (GM, open circles). Regression line calculated by method of least squares and drawn for all data together ($r = 0.964$, $n = 24$, $P < 0.001$). Each data point represents the calculations made for one proximo-distal level in one individual muscle (totally three levels in each one of four ED and four GM muscles; see Methods for further information). (B) Average frequency of type I fibres (%) vs. proximo-distal level for ED (filled triangles) and GM muscles (open circles). Means ± SE (some of the SE-bars approach the size of the plot symbols). Same muscles and material as in panel (A). In each muscle, the difference between the most proximal and the most distal mean value was statistically significant (t test, $P < 0.05$ or better).

The GM and ED muscles differed with regard to how the sizes of different fibre types changed with proximo-distal level (Table 2). Within ED, the midlevel type I fibres (level 4) were significantly larger than those of level 2 or 6. For GM, there was rather a tendency for the type I fibres to become progressively thinner at more distal levels (significantly smaller at level 6 than at level 2).

3B).

Degree of regionalization of type I fibres at different proximo-distal levels

In relation to the cross-sectional muscle area, the regions containing the type I fibres were different in different muscles being, for instance, relatively large in PE and small in TA (Figure 4A). However, in all the investigated muscles we found a strikingly similar pattern of a proximal-to-distal decrease of the relative size of the type I fibre region (Figures 1, 4A-B). This behaviour was statistically highly significant for each one of the five muscles (FRs, Table 1).

The type I fibres became progressively more eccentrically localized within cross-sections taken at more distal levels (Figure 1). Correspondingly, the relative length of the “type I fibre vector” (arrows in Figure 1) became progressively larger at more distal levels (Figure 4C and D). The correlations between relative vector length and proximo-distal level were statistically significant for all muscles except GM (Table 1). Also in this latter muscle, however, the relative vector length was significantly greater for the two most distal levels than for the two most proximal ones (t test, $P < 0.001$).
Fig. 4. Differences in type I fibre regionalization between cross-sections taken at different proximo-distal levels. Same symbols and muscles as in Figure 2, mean values (in A and C) ± SE plotted vs. proximo-distal level. (A) Type I fibre region, given in % of total cross-section area. The region containing type I fibres (cf. interrupted lines in panels of Figure 1) was delineated using the “sector method” (see Methods). (B) Values of panel A, normalized (%) vs. maximum mean value for each muscle. (C) Length of the “type I fibre vector”, connecting the centre of mass for the muscle cross-section to that for only the type I fibres (given in % of the equivalent diameter of the muscle cross-section; cf. arrows in panels of Figure 1). (D) Vector length values of panel C, normalized (%) vs. maximum mean value for each muscle. The vector length gives a measure of the degree of type I fibre eccentricity within the muscle section.

Fig. 5. Differences in the direction of type I fibre regionalization at different proximo-distal levels. Means ± SE for the direction of the “type I fibre vector” connecting the centre of mass for the muscle cross-section to that for only the type I fibres (degrees). Zero or 360° degrees is medial, 90° is posterior, 180° is lateral and 270° is anterior. (A) Data for the three muscles in which the vector angle remained relatively constant (ED, FD, TA); for ED there was actually a small but statistically significant increase of vector angle from proximal to distal (cf. Table 1). (B) Data for the two muscles with marked proximo-distal shifts in the vector angle (GM, PE). For PE, the difference between the mean angles for levels 1 and 2 was statistically significant (t test, P < 0.02). For GM, see Table 1.
Direction of type I fibre regionalization at different proximo-distal levels

Within cross-sections, the direction of type I fibre regionalization was quantified by determining the direction of the type I fibre vector (see Methods). This parameter, the “vector angle”, behaved differently for different muscles. For ED, FD and TA, the vector angle remained almost the same at different proximo-distal levels (Figure 5A). This does not mean that there were no variations at all; for ED there was actually a small but statistically significant increase of vector angle from proximal to distal (about 30°; Table 1, Figure 5A). Much larger rotations, by about 100°, were seen in the two remaining muscles: in GM there was a progressive decrease of the vector angle and in PE the vector angle increased by about the same amount, being changed most markedly between the two most proximal levels (Figure 5B). At distal and midlevel muscle portions, both muscles had their type I fibres oriented roughly in the direction of the centre of the limb (Wang and Kernell, 2000), the arrow pointing antero-medially for PE and in an anterior direction for GM. Proximally, however, this was not the case: here the type I fibres of GM were accumulated toward the medial (outer) muscle rim and those of PE towards antero-lateral muscle portions. For GM, this tendency is further illustrated in Figure 6B and D. As these

![Figure 6](image)

**Fig. 6.** Proximo-distal shift in the orientation of type I fibre regionalization within both heads of gastrocnemius. Digitized cross-sections from gastrocnemius lateralis (A, C) and medialis (B, D) at proximo-distal level 1 (A, B) and 4 (C, D). Level 1 is the most proximal one and level 4 is at the lengthwise middle of the muscle (“midlevel”). All sections from same limb.

<table>
<thead>
<tr>
<th>Muscle portion:</th>
<th>ED</th>
<th>FD</th>
<th>GM</th>
<th>PE</th>
<th>TA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximal-I&amp;II</td>
<td>11.9±1.0</td>
<td>5.8±0.3</td>
<td>9.8±2.2</td>
<td>6.9±0.6</td>
<td>12.6±0.8</td>
</tr>
<tr>
<td>Proximal-II</td>
<td>12.6±1.1</td>
<td>8.7±0.6</td>
<td>9.6±2.9</td>
<td>7.1±0.6</td>
<td>14.5±0.7</td>
</tr>
<tr>
<td>Distal-I&amp;II</td>
<td>12.7±0.7</td>
<td>5.4±0.3</td>
<td>10.4±1.7</td>
<td>6.4±0.5</td>
<td>12.4±0.5</td>
</tr>
<tr>
<td>Distal-II</td>
<td>12.7±1.3</td>
<td>8.2±0.3</td>
<td>10.5±1.5</td>
<td>6.7±1.2</td>
<td>14.3±0.4</td>
</tr>
</tbody>
</table>

Means ± SD for fibre lengths (mm) as measured from different sampling sites in each muscle. Each value is the mean of averages from five different limbs (three rats). Within each individual muscle, about 20 dissected fibres were measured for each sampling site.
findings are not in accordance with general views on the type I fibre regionalization of gastrocnemius (but confirming observations on GM by DeRuiter et al., 1996), we also made a more restricted investigation of these properties for the lateral head of gastrocnemius (GL). As is illustrated in Figure 6A and C, the proximo-distal change in type I fibre regionalization in GL was largely a mirror image of that in GM; in proximal portions of both muscles, type I fibres were prominently present in superficial (non-deep) muscle portions.

### Table 4. Fibre lengths and pinnation angles

<table>
<thead>
<tr>
<th>Muscle</th>
<th>ED</th>
<th>FD-I&amp;II</th>
<th>FD-II</th>
<th>GM</th>
<th>PE</th>
<th>TA</th>
</tr>
</thead>
<tbody>
<tr>
<td>FibLen (mm)</td>
<td>12.5±1.0</td>
<td>5.6±0.4</td>
<td>8.5±0.5</td>
<td>10.1±2.0</td>
<td>6.8±0.8</td>
<td>13.5±1.2</td>
</tr>
<tr>
<td>Mlen (mm)</td>
<td>28.2±0.8</td>
<td>27.2±1.2</td>
<td></td>
<td>32.3±0.6</td>
<td>23.4±0.8</td>
<td>24.7±1.2</td>
</tr>
<tr>
<td>PenAng (deg)</td>
<td>8</td>
<td>21 (a), 34 (p)</td>
<td>16</td>
<td>26</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>FibExt (mm)</td>
<td>12.3</td>
<td>7.5</td>
<td>9.7</td>
<td>6.1</td>
<td>12.6</td>
<td></td>
</tr>
<tr>
<td>FibExt/Mlen (%)</td>
<td>43.8</td>
<td>18.3</td>
<td>27.7</td>
<td>30.0</td>
<td>26.1</td>
<td>51.2</td>
</tr>
<tr>
<td>FibExt / Level</td>
<td>3.1</td>
<td>1.3</td>
<td>1.9</td>
<td>2.1</td>
<td>1.8</td>
<td>3.6</td>
</tr>
<tr>
<td>Mwt (mg)</td>
<td>152</td>
<td>392</td>
<td>713</td>
<td>145</td>
<td>633</td>
<td></td>
</tr>
<tr>
<td>Physiological CSA (mm²)</td>
<td>11.3</td>
<td>45.6</td>
<td>63.4</td>
<td>17.9</td>
<td>41.3</td>
<td></td>
</tr>
<tr>
<td>“Relative force capacity”</td>
<td>7.4</td>
<td>11.6</td>
<td>8.9</td>
<td>12.3</td>
<td>6.5</td>
<td></td>
</tr>
</tbody>
</table>

**Abbreviations:** FibLen, fibre length; Mlen, muscle length; PenAng, pinnation angle; FibExt, proximo-distal fibre extent; Mwt, average muscle weight (from Wang and Kernell, 2000); CSA, cross-section area; FibExt / Level, FibExt divided by (Mlen / 7), i.e. by the interval between consecutive proximo-distal analysis levels (cf. Figure 1). Means for FibLen and Mlen are shown ± SD (five muscles). FibExt was equal to (FibLen x cos(PenAng)), which corresponds to the proximo-distal extent of the respective muscle fibre within the muscle. The maximum number of consecutive analysis levels (Figure 1) within which a given fibre may be seen is equal to ((FibExt / Level) + 1). For calculation of physiological CSA, see Methods. For FD, anterior (a) and posterior (p) pinnation angles are given and the calculations for FibExt and CSA were made using the average for both angles. Similarly, calculations of physiological CSA were for FD made using average values of fibre length (mean of FD-I and II and FD-II). “Relative force capacity” calculated as 100 x (CSA / Mwt).

**Fibre lengths and pinnation patterns vs. fibre type regionalization**

A length-wise change in the density and distribution of type I fibres (Figures 1-6) might depend on alterations of single fibre properties and/or on shifts in fibre populations along the muscle. Hence, for the interpretation of the present results it is essential to know how long the individual fibres are in the various muscles and how they tend to be spatially organized.

Using macerated muscles, we measured the lengths of single dissected fibres within each one of the five muscle spe-
As we were now primarily interested in the transverse and longitudinal regionalization features of the type I fibres, we choose to sample fibre lengths in type I and II vs. type II-only regions for each one of two proximo-distal levels (Table 3). Statistically significant differences in fibre length were in almost all cases observed between the four sampling regions for each individual muscle (ANOVA, P < 0.05 or better in 23 of the 25 analyzed muscles). However, in most cases these regional differences in fibre length were relatively small; FD was the only muscle species for which individual regional samples differed by more than 8% from the average for all samples together (cf. Tables 3 and 4). Hence, in the further analysis we pooled all fibre length measurements for each muscle except FD.

For this latter muscle we made a provisional distinction between “type I and II” and “type II-only” muscle portions, each one with “anterior” and “posterior” pinnation angles (a) and (p), Table 4. The angle of fibre pinnation and the approximate organization of tendons and aponeuroses were determined using dissections of fresh muscles (three samples for each muscle species). These observations and the measurements of fibre length are summarized in Table 4 and in the schematic drawings of Figure 7. As single fibres would often be represented in two or more of the seven consecutive “analysis levels” of each muscle (cf. Figure 1), pinnation patterns may have contributed to gradual proximo-distal shifts in type I fibre localization in the transverse plane. This seems likely to have been the case for the changes in vector angle seen in PE and GM. In PE, the initial shift of type I fibre accumulation from antero-lateral to antero-medial (Figure 5B) may (partly) have reflected the intra-muscular course of type I fibres originating anterolaterally in the proximal tendon sheet and inserting more medially at the distal tendon (Figure 7, PE). Similarly, in GM the proximo-distal shift in type I fibre accumulation from antero-medial to antero-lateral...
lateral (Figures 5B, 6B and D) might (partly) have reflected the intra-muscular course of its type I fibres originating from the medial-proximal tendon sheet and inserting at the lateral-distal sheet (Figure 7, GM). Corresponding conditions might also have been underlying the shifts of type I fibre localization in gastrocnemius lateralis (Figure 6A and C). The architecture of the latter muscle seemed, however, to be considerably more complex than that of GM.

With the exception of TA, all the muscles had mean fibre lengths that were less than half that of the muscle, i.e. in most of the investigated muscles no single cross-section would show all the fibres (Table 4). The number of consecutive proximo-distal “analysis levels” (cf. Figure 1) within which the same single fibres might be represented was 1-2 for FD and PE, 2-3 for GM, and 3-4 for ED and TA (Table 4, “FibExt / Level”). Thus, the marked and progressive proximo-distal changes in type I fibre density and regionalization across the seven levels of analysis (Figures 2 and 4) is likely to have been reflecting changes in the composition of fibre populations present at different proximodistal levels. Further measurements are, of course, needed for establishing whether proximo-distal changes of myosin composition also took place within individual muscle fibres (cf. Edman et al., 1985; Edman et al., 1988; Sakuma et al., 1995).

Discussion

Proximo-distal type I fibre distribution

The present results represent the so far most extensive systematic analysis of general patterns of fibre type distribution in a proximo-distal direction. In previously published work from other laboratories, individual muscles or muscle compartments have only infrequently been studied with regard to the proximo-distal fibre type distribution, and few direct comparisons have been made between different muscles in this respect. No studies have been made of how the fibre type regionalization within cross-sections changes along the proximo-distal axis (Figures 1, 4 and 5). In the cat, a decline of type I fibre density from proximal to distal was seen within a neuromuscular compartment of the lateral gastrocnemius (data from English and Letbetter, 1982; see plot in Kernell, 1998) and within parts of peroneus longus (Donselaar et al., 1987). In the rat, a decrease of type I fibre frequency from proximal to distal has been measured for GM (Gardiner et al., 1991) and for ED (Punkt et al., 1998). However, the opposite tendency was seen for the slow soleus in which the type I fibres increased in frequency from proximal toward distal (Punkt et al., 1998). It is important to stress that, although all the five “fast” hindlimb muscles of the present study showed a remarkably similar pattern of proximo-distal type I fibre organization (Figures 2D, 4B and D), the direction of such proximo-distal differences might conceivably differ between different species, limb portions and muscle types. This caution gets further underlined by the fact that, in other preparations than that of the rat’s lower hindlimb, an increase of type I fibre density from proximal to distal (i.e. qualitatively opposite to the present patterns) have also been observed for some “fast” muscles (rabbit’s extensor digitorum lon-
gus, Lexell et al., 1994; young cattle semitendinosus Brandstetter et al., 1997).

In our preceding study of midlevel data, only a modest negative correlation was found between the overall type I fibre density and the degree of type I fibre eccentricity (Wang and Kernell, 2000). The co-variation between the overall fibre density and measures of regionalization were more evident in the present proximo-distal comparisons (cf. Figures 2D, 4B and D). Although not self-evident, it is intuitively understandable that regionalization might become less distinct for large than for smaller relative populations of the target fibre types. In the muscles studied here, the midlevel proportion of type I fibres typically varied between about 3 and 13 % (cf. Figure 3). Substantially larger type I fibre regions (and smaller degrees of type I fibre eccentricity) would be expected for human muscles (type I fibre proportion often around 50%) than for the present rat muscles. Correspondingly, published data for human muscles have so far demonstrated the common presence of vector-regionalization but not any appreciable degree of area regionalization (e.g., Johnson et al., 1973; Lexell et al., 1983).

Possible mechanisms and consequences

Why would type I fibres tend to be more common proximally than more distally within single muscles of the rat lower hindlimb? Was this tendency related to particular functional properties or tasks of these muscles? In this context it is interesting to note that, from various functional points of view, the five studied muscles were widely different. Using the measurements of fibre length and pinnation angle, we calculated the extent to which the present five muscles were
specialized for large degrees of shortening or for great amounts of active force production. The “relative shortening capacity” was calculated as the ratio between “fibre extent” and muscle length, where fibre extent was equal to the extent of single fibres along the axis of whole-muscle shortening. The “relative force capacity” was calculated as the ratio between the physiological cross-section area (CSA) of the muscle and its weight (for calculations, see Table 4). As expected, the two parameters show a reciprocal relationship. The TA and ED muscles were those most specialized for shortening and PE and FD were more specialized for force production with GM occupying an intermediate position. The overall density of type I fibres, which is likely to be high in muscles with pronounced postural functions, was relatively low in the two “high-shortening” muscles (TA, ED) but also in one of the “high-force” muscles (FD; see Figure 2C). All the five muscles showed an almost identical pattern of proximo-distal type I fibre regionalization (Figure 2D). Thus, this latter kind of organization was apparently largely independent of whether muscles were specialized for shortening contractions, for force production or for prolonged postural functions.

The slow type I fibres largely rely on oxidative metabolism and have to be well provided for by the local circulation. During contractions, circulation may become impeded by an increase of intramuscular pressure. It has been demonstrated that this pressure increase may show huge differences within single muscles, depending on the curvature of the muscle fibres and the curvature, shape and tension of the tendon sheets (Otten, 1988). It should be investigated whether, in the rat’s lower hindlimb, such pressure increases might be less pronounced in proximal vs. in more distal muscle portions. However, among the present five muscles, a general proximo-distal difference of this kind seems rather unlikely because several of the muscles had a central distal tendon, an arrangement which would not be expected to be associated with large pressure transients (Otten, 1988).

Another factor of possible relevance might be the thermal balance of deep proximal and distal muscle portions. Muscle fibres get an increased shortening speed and, thereby, an increased power as a result of the temperature increase caused by intense muscle activity (e.g. Sargeant, 1987). Such heat increases would be expected to be more marked for central than for more superficial limb portions, i.e. these effects on power would be particularly noticeable for slow muscle fibres, thanks to their central intra-limb position (e.g. Armstrong and Phelps, 1984; Wang and Kernell, 2000). During intense locomotor activity, the heat-enhancement of power would help to make the slow fibres more useful companions for the fast ones in the generation of concentric muscle contractions. In a small-sized animal like the rat (high surface-area : volume ratio), the “deep” intramuscular temperatures might conceivably become more easily elevated in proximal than in the tapering, more distal portions of the hindlimb. Hence, in such animals it might be advantageous for the heat-enhancement of power to have slow fibres accumulated in proximal muscle portions (Figure 2D).

Alternatively, the proximo-distal as well as the cross-sectional aspects of the
type I fibre regionalization might (partly) reflect organizational consequences of processes during the initial development of the limb (cf. discussion in Wang and Kernell, 2000). In some cases, proximo-distal differences in type I fibre frequency were seen during development but not in the (more) adult state (rat soleus, Sakuma et al., 1995; cattle semitendinosus, Brandstetter et al., 1997). For the interpretation of the present findings it would be essential to know how the regionalization of fibre types in a transverse plane, as seen during early embryological development (e.g., Narusawa et al., 1987; Condon et al., 1990), is related to the (presumably) later development of a pinnate muscle structure (cf. Stickland, 1983). For instance, which factors cause certain portions of the primordial muscle to become shifted toward more distal positions within a pinnate muscle?

Whatever the reasons and functional consequences of the lengthwise fibre type regionalization, the present findings are also relevant as a background for further physiological investigations of rat’s hindlimb muscles and of problems concerning the differentiation and innervation of different muscle fibre types. When investigating the fibre type composition of hindlimb muscles, great care must be taken to identify the proximo-distal level(s) from which the fibre samples were obtained (cf. Figure 2C and D). In previously published literature, widely varying figures appear for the fibre type composition of corresponding muscles and the proximo-distal level of sampling was typically defined only very approximately (e.g. as the “muscle belly”) or not at all.

References


