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Multiple methods for quantifying the spatial distribution of different categories of motoneuronal nerve endings, using measurements of muscle regionalization

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Abstract

For skeletal muscles, a well-known match exists between the properties of motoneurones and those of their muscle fibres. Hence, the intramuscular distribution of different kinds of motoneuronal nerve endings (e.g. “slow” versus “fast”) can be mapped by determining the distribution of the corresponding types of muscle fibre. As a background for further studies of motoneuronal plasticity, we needed precise measures of such distributions. Simple quantitative methods were developed for defining the position and extent of sub-populations of cells within a structure (e.g. the regional distribution of slow versus fast muscle fibres within a muscle cross-section): (a) The “mass vector method” defined the relative position of the target cell cloud. A line was drawn between the calculated centre of mass for the target cells and that for the whole structure. The direction (a1) and length (a2) of this line gave a measure of the direction and degree of target cell eccentricity within the structure. (b) The “sector method” delineated the region containing the target fibres. A circle around the centre of mass for the target fibres was subdivided into a number of equal sectors (standard setting: 20). The most remote point was found within each sector and a line joining these points defined the region of the target fibres. When applied to the “slow” type I fibres of cross-sections from rat hindlimb muscles, the regional area estimates obtained by the sector method were highly correlated with, but about 10% lower than those achieved by the well-established “convex hull” method. Highly significant inter-muscular differences were observed for each one of the three new parameters described in this paper (a1, a2, b).

Key-words: Cell distribution; Motor nerve endings; Skeletal muscle; Fibre types; Rat; Convex hull

1. Introduction

For skeletal muscles, a well-known match exists between the properties of motoneurones and those of their muscle fibres (Burke, 1981; Kernell, 1992). Hence, the intramuscular distribution of different kinds of motoneuronal nerve endings (e.g. those of “slow” versus “fast” motoneurones) can be mapped by determining the distribution of the corresponding types of muscle fibre. In the context of studies concerning the organization and plasticity of spinal motoneurones, we were interested in how well the intra-muscular distribution of different kinds of motoneuronal terminals would be restored after denervation and subsequent re-innervation (Wang and Kernell, 1999). For the performance of this analysis, we needed to measure the spatial distributions of “fast” and “slow” cells with a sufficient degree of precision. Hence, we developed a set of simple methods for defining the position and extent of sub-populations of cells within a structure, i.e. in our case the regional distribution of slow versus fast muscle fibres within a muscle cross-section. To describe and evaluate these methods form the main objectives of the present paper.
2. Muscle fibre types and “regionalization”

Histochemically, muscle fibres are often categorized as types I or II on basis of their staining properties for myofibrillar ATPase (mATPase; Brooke and Kaiser, 1970). After, for instance, acid preincubation, type I fibres are dark and type II fibres pale. Combined histochemical and physiological studies have demonstrated that the type I fibres are more slowly contracting than the type II fibres (Burke, 1981). At least in some limb muscles, the regional distribution of the two main types of muscle fibre are markedly different. In a section through the muscle belly of, for instance, gastrocnemius medialis or tibialis anterior, the slow type I fibres tend to be accumulated in deep muscle regions (Kernell, 1998). Although it has since long been known that such “fibre type regionalization” exists (implying also a corresponding “regionalization” of motoneuronal nerve endings; see above), the phenomenon has not received much systematic attention. Studies have been limited to only a few muscles, and regional differences in fibre distribution have typically been indicated by measurements of the percentages for different fibre types in limited samples obtained from one or a few deep and one or a few superficial muscle regions (e.g. Johnson et al., 1973; Armstrong and Phelps, 1984). In some cases, more graded and continuous measurements of fibre distribution have been tried; thus, Pullen (1977a, b) counted the percentages of different fibre types along imaginary guide-lines drawn through the muscle section in a dorso-ventral or medio-lateral direction. The previously used methods give detailed or pointwise descriptions, but they do not provide general figures for the degree and/or direction of fibre type regionalization. Other, complementary methods are needed for answering spatial questions like: How much does the centre for a given fibre type population differ from that for the muscle as a whole? How much of the available cross-section space of a muscle is utilized by a given fibre type? Techniques for analyzing such questions are described below.

3. Experimental procedures

Our investigation concerned the distribution of type I fibres within muscles of the lower hindleg of adult rats. Under general anaesthesia (pentobarbitone, 50 mg/kg i.p.), the muscles were gently freed from the surroundings, identified and provided with a labelling stain on their posterior and/or lateral sides. The muscles were fixed by freezing in isopentane cooled by liquid nitrogen. Cross-sections of 10 µm were cut in a cryostat and stained for mATPase after acid preincubation (pH 4.3; Brooke and Kaiser, 1970). For results to be used in the present context, the sections were taken from middle portions of the respective muscles, at about the same distance from the first muscle-free pieces of the proximal and distal tendons.

Using a microfiche-copier (Canon PC Printer 70M), low-magnification prints were obtained of total cross-sections of each muscle; a magnification of about 47x was typically sufficient for clearly distinguishing each single type I fibre. Using these high-contrast paper prints, the muscle outline and the site of each stained type I fibre was entered into a PC using a graphic tablet and custom-
made software. The data were saved as X-Y coordinates in an ASCII-file that could be read by custom-made programs for further data processing. For the purpose of illustration, the data were also converted to files in HPGL format that could be read and printed with common graphic software (e.g. CorelDraw). Examples of such illustrations are shown in Figs. 1 and 2.

The spatial analysis-methods were illustrated and validated using measurements obtained from five different hindlimb muscles (extensor digitorum longus, ED; gastrocnemius medialis, GM; peroneus brevis, PB; peroneus longus, PE; tibialis anterior, TA), each muscle being studied in at least six rats. The presence of significant differences between results from different muscles was demonstrated using variance analysis (ANOVA).

Fig. 1. (A) Muscle outline and positions of type I fibres, as traced from cross-section through middle of extensor digitorum longus muscle (ED). “Mass vector” (“target fibre vector”) indicated by arrow with its tail at the centre of mass for the muscle section and its head at the centre of mass for the cloud of type I fibres (see Text for further explanation). (B-C) Bar graphs (means ± SE) illustrating how the mass vector measures differed between rat hindlimb muscles, as shown for: gastrocnemius medialis (GM, n = 7), tibialis anterior (TA, n = 6), ED (n = 7), peroneus longus (PE, n = 8) and peroneus brevis (PB, n = 8). (B) Relative length of the mass vector for type I fibres (Vector length, % of equivalent muscle section diameter). (C) Direction of this vector (Vector angle, degrees).
4. Measures of “regional eccentricity”

Looking at Fig. 1(A), it is obvious that the cloud of type I fibres had an eccentric position within the muscle. As a method for quantifying the position of a “fibre-cloud” we calculated its fictive centre of mass. The same calculations were made for the muscle cross-section as a whole. The arrow drawn into Fig. 1(A) runs from the centre of mass of the muscle and points at the centre of mass for the type I fibres. The length and direction of this vector gives a measure of the direction and degree of regional eccentricity for the type I fibres. Some further details are given concerning these calculations below.

4.1 Fibre “centre of mass” (FiCeMa)

For these calculations, each one of the type I fibre profiles was assumed to have the same mass. The centre of mass was calculated in a simple and straight-forward cumulative manner, using standard equations for forces acting on lever arms (i.e. inter-fibre distances). First, the centre of mass was calculated for fibre 1 ver-
sus 2, then that for (1+2) versus 3, etc.

4.2 Muscle “centre of mass” (MuCeMa)

For these calculations, all portions of a cross-section were assumed to have the same thickness and specific weight. With the exception of prominent intra-muscular tendons, all of the muscle section was included in the calculations. Each muscle cross-section was subdivided into 100 parallel stripes of a uniform width but with a variable length (depending on the muscle outline). Firstly, the centre of mass was determined for each stripe (i.e. its midpoint). Secondly, cumulative calculations like those for the muscle fibres (see above) were used for obtaining the centre of mass for the whole muscle section.

4.3 Normalization of vector direction and length

Muscle cross-sections were processed with their lateral side to the left and the dorsal (posterior) side at the top. Thus, according to standard trigonometric conventions, a regionalization arrow (“target fibre vector”) pointing due right (i.e. in a straight medial direction) would have an angle of 0° (or 360°). The mass-vector of Fig.1(A) has, for example, an angle of 354°.

For comparisons between muscles, the length of each target fibre vector was normalized in relation to muscle size. The surface area of the muscle cross-section was calculated from the traced outline. From this area, an “equivalent muscle diameter” was calculated, being the diameter of a circle with the same area as that of the muscle cross-section. The length of the target fibre vector was expressed as a percentage of the equivalent muscle diameter.

There were reproducible and highly significant differences between the various hindlimb muscles in the direction and relative length of their target fibre vector (ANOVA, P < 0.001). Thus, the determinations of vector angle and length were both sufficiently precise for demonstrating marked and statistically significant differences in these measures between different hindlimb muscles (Fig.1(B-C)).

5. Measure of “regional restriction”

In most of the studied hindlimb muscles, the type I fibres were distributed within only part of the total cross-section (Fig.1(A)): some regions consisted wholly of type II fibres whereas other portions contained a mixture of type I and II fibres. We used two methods for the (semi-)automatic delineation of the region containing the type I fibres (“type I fibre region”):

1. the well-established method of the “convex hull”, which is described in standard books on computer algorithms (e.g. Cormen et al., 1990). Similar methods (often manually applied) have been used in several studies for delineating the territories of single muscle units (e.g. Pierotti et al., 1991; Rafuse and Gordon, 1996).

2. an alternative method, developed during the course of this work (“sector method”).

The outcome of the two methods for delineating the type I fibre region is illustrated in Fig.2. The line calculated according to the convex hull method encloses the cloud of points as if the line were a string tightened around a corresponding set of fixed nails (Fig.2(A)); as the name
indicates, this means that nowhere around the circumference of the cloud does this border line show an inward concavity of the kind that one might be tempted to draw for irregular cloud structures like the one of Fig.1(A). In order to accommodate such irregular shapes, we developed the alternative “sector method” for delineating the cloud of type I fibres (Fig.2(B-C)).

The sector method calculations had as their starting point the target fibre centre of mass described above (FiCeMa). A circle covering the whole muscle cross-section was drawn with its centre at FiCeMa. This circle was subdivided into \( n \) sectors of equal angles. Within each sector, the most distant fibre was identified. The type I fibre region was enclosed within the lines connecting all these most-distant fibres (Fig.2(B-C)).

For comparisons between muscles, the area of the type I fibre region was normalized as a percentage of the muscle cross-section area. Areas calculated using the convex hull method were, on average, about 10% larger than those obtained by the sector method. Both measurements were very highly correlated (Fig.3(A)). Whichever method was used, reproducible and highly significant differences were found between the various hindlimb muscles in their relative type I fibre region (ANOVA, \( P < 0.001 \); Fig.3(B)). The estimate of the type I fibre region often looked somewhat more realistic with the sector method than with the convex hull method.

In contrast to the convex hull method, the sector method is semi-automatic: the experimenter must make a decision concerning the number of sectors to be used for the calculations of the type I fibre region. For Fig.2(B-C), the calculations were made using our standard setting of 20 sectors. Provided the total number of type I fibres was not too low (i.e. at least above about 60), this setting usually gave intuitively satisfactory results, resembling the outlines that an experimenter would be tempted to draw by hand. The method often caused a few fibres to fall just outside the enclosed region; in terms of the
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regional area estimate these “errors” were, however, typically negligible when using 15 sectors or more. For the muscles of Figs.1(B-C) and 3, the sector method was found to be relatively insensitive to minor changes in the number of sectors. In a subset of 15 muscles (three each of ED, GM, PB, PE and TA) the mean type I fibre region was 56.0 ± 23.7 (SD) % for 15 sectors, 54.6 ± 22.9 % for 20 sectors and 53.8 ± 23.0 % for 25 sectors. An extreme lowering of the number of sectors would, of course, cause many fibres to fall well outside the enclosed region. Similarly, an extreme increase in the number of sectors would give a fragmented and “artificial-looking” type I fibre region (Fig.2(D)). For small cell populations (< about 60), the number of sectors should be decreased below 20 (e.g. always keeping the number of sectors below about a third of the number of fibres).

6. Conclusions and comments

The spatial position and extent of cell/fibre populations constitute important aspects of the organization of muscles and their motor nerve endings. The analysis of such features might be further optimized, we think, with quantitative techniques such as those introduced in the present article. However, the applicability of methods such as those discussed in the present article is not limited to peripheral neuro-muscular contexts, but these techniques would be of potential interest also for many issues concerning the central organization of the nervous system. Within the brain and spinal cord, neurones of different types are often topographically organized such that cells of a given property or connection pattern occur preferentially within a given region. Typical examples include the intraspinal distribution of motoneuronal cell bodies: cells innervating different neighbouring muscles are partly segregated into different regions within the ventral horn (Romanes, 1951; Vanderhorst and Holstege, 1997). Quantitative methods for defining such regional cell distributions would, for instance, make it easier to analyze the degree to which the motoneurones of particular (e.g. synergistic) muscles may differ in their intra-spinal localization.

References

Vanderhorst VG, Holstege G. Organization of lumbosacral motoneuronal cell groups innervating hindlimb, pelvic floor, and axial muscles in the cat. J Comp Neurol, 1997; 382: 46-76.