Regional organization of fibre types in normal and reinnervated hindlimb muscles
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CHAPTER 8

Summary and conclusions
The contractile elements of skeletal muscles, the muscle fibres, can be equipped with widely different biochemical and physiological properties. Different kinds of muscle fibre are optimally suited for different motor tasks. Fibres with slow contractions and a high resistance to fatigue are appropriate for use in long-lasting postural contractions, and fast fibres with a high power are suitable for producing brisk shortening contractions in movements. Within single limb muscles, fibres of both these main kinds are typically present, albeit in different proportions for different muscles. The “slow” and “fast” fibres can be recognized in histological preparations stained for myofibrillar ATPase (mATPase), then referred to as type I and II fibres (see Chapter 1).

The present thesis concerns the spatial distribution of the “slow” type I fibres within muscles of the hindlimb. It is known since long ago that some muscles may have strikingly heterogeneous distributions of type I and II fibres, but this phenomenon of “fibre type regionalization” has still not attracted much quantitative analysis and is not well understood.

In the present thesis, new methods and procedures are described for the quantification of fibre type regionalization in histochemically stained preparations. These methods have been applied to muscles of the lower hindlimb in three species of common laboratory animals (mouse, rat, and rabbit). The normal patterns of regionalization have been compared to those found after denervation and reinnervation. Below, various aspects of these investigations are described in somewhat greater detail.

In Chapter 2, newly developed methods are presented for the quantification of different aspects of fibre type distribution within a muscle section. Firstly, a “mass vector method” is described which defines the position of a collection of fibres in relation to the muscle cross-section. In this procedure, the centre of the muscle section is calculated as the centre of mass for a uniform sheet of the same shape and size. Furthermore, using standard rules of mechanics, the centre of mass is calculated for a collection of fibre-site-indicators, all with the same mass. The “target fibre vector” (e.g., “type I fibre vector”) is the line connecting the centre of mass for the muscle section with that for the target fibre-sites. The direction of this vector indicates the direction of any eccentricity of target fibre position, and the relative length of the vector gives a measure of the degree of eccentricity.

Secondly, methods are explored for the delineation and quantitative assessment of the region containing the target fibres (e.g. the type I fibres). One of these methods, the “sector method”, was newly developed for this project. Results obtained using the sector method were compared to those produced with the well-known method of the “convex hull”. The “sector method” followed the distribution profile of the target fibres in somewhat greater detail whereas the “hull method” had the advantage of being more automatic and less dependent on preset parameters.

In Chapter 3 the new procedures were applied to a study of fibre type regionalization in all the ankle-traversing muscles of the rat’s lower hindlimb. The following 12 muscles were studied for cross-sections taken at the proximo-dis-
tal middle (muscle “belly”): ED, extensor digitorum longus; EH, extensor hallucis longus; FD, flexor digitorum & hallucis longus; GL, gastrocnemius lateralis; GM, gastrocnemius medialis; PB, peroneus brevis; PD, peroneus digits 4 & 5; PE, peroneus longus; PL, plantaris; SO, soleus; TA, tibialis anterior; TP, tibialis posterior. With the exception of the “slow” SO, all these muscles are dominated by fast fibres (“fast” muscles). A distinction was made between two potentially independent aspects of spatial fibre-distribution: “area-regionalization” and “vector-regionalization”. The former is a measure of extent to which the target fibre type is restricted to a limited area within a muscle cross-section, as quantified using the sector- or hull-method for defining the target fibre region (FRs, FRh). In these measurements, the target fibre region is expressed as a percentage of the total cross-section area of the muscle. The degree and direction of “vector-regionalization” is given by the relative length (VL, in percent of muscle diameter) and direction (VA) of the target fibre vector, i.e. it is related to the degree and direction of target fibre eccentricity within the muscle section. All the muscles were vector-regionalized and most of the fast muscles were also area-regionalized (exceptions: PB, PD). Furthermore, these two aspects of regionalization were strongly correlated. The direction and degree of vector-regionalization were both significantly related to the position of the respective muscles within the limb: within each muscle cross-section type I fibres typically (but not always) tended to be accumulated toward the centre of the limb and the degree of intra-muscular fibre type eccentricity was greater for muscles situated far from the limb-centre than for those placed more centrally.

In **Chapter 4** the investigations were extended to a length-wise analysis of muscle composition. This was done for a subset of five “fast” muscles of the rat’s hindlimb (ED, FD, GM, PE and TA), each one being analyzed at 7 proximo-distal levels. From a general point of view, the five investigated muscles had markedly different motor tasks and functional specializations (e.g., different proportion of type I fibres, architectural features, etc.). Still, a surprisingly uniform proximo-distal pattern of type I fibre distribution was found: in all the muscles there was a similar and marked decline of type I fibre density from proximal toward more distal levels. This decline in overall type I fibre density was associated with a similarly stereotyped increase in the degree of area- and vector-regionalization from proximal toward distal. As a background for the interpretation of these findings, measurements were also made of key architectural features of the five muscles: fibre length was determined for single fibres that were dissected from macerated muscles, and the approximate angle of fibre pinnation was measured in dissected fresh preparations. All the five muscle species had a pinnate structure and the relative fibre length varied, on average, from 21 to 55 % of total muscle length. Thus, the length-wise variation in type I fibre density might well have been caused by a lengthwise change in the proportion of the different fibre types; the results could be explained without assuming the existence of length-wise changes of properties within single fibres.

In **Chapter 5** the findings for rat hindlimb muscles were compared to those
from two other species of commonly used laboratory animals: rabbit and mouse. In general, similar patterns of type I fibre regionalization were observed in all the three animal species, all muscles being vector-regionalized and most of them also area-regionalized. There were, however, striking and consistent species-related differences with regard to the degree of both aspects of regionalization in “fast” hindlimb muscles. Thus, the relative size of the type I fibre area was ranked such that rabbit > rat > mouse, and the degree of type I fibre eccentricity (relative vector length) ranked, correspondingly, rabbit < rat < mouse. These species-associated differences took place independently of the overall density of type I fibres in the respective muscles. In the lengthwise direction, the proximo-distal changes of type I fibre density were, for most muscles, little prominent in the rabbit but relatively similar between rats and mice.

The results of the comparisons between the three animal species, showing the existence of clear species-associated differences, suggested that patterns of fibre type regionalization might be adapted to functional requirements rather than being stereotyped remnants of processes for embryological development. The functional advantages of having a regionalized distribution of type I and II fibres are still unclear; various alternative possibilities were discussed in Chapters 3-5.

The processes responsible for the emergence of fibre type regionalization should include, during the embryological period of muscle differentiation, mechanisms for guiding the axons of “slow” motoneurones toward the regions containing the future “slow” muscle fibres. In Chapter 6 experiments were described for testing whether such mechanisms for axonal guidance are active also in the adult animal. In adult rats, the sciatic nerve was sectioned and re-united at the level of the upper hindlimb. Following such an operation, the distal portion of the axon will degenerate, leading to muscle denervation. Axons of the proximal stump of the nerve will, however, grow out again and reinnervate any denervated muscles they may encounter. It is known from earlier studies that regenerating motor axons are incapable of finding or recognizing their own species of muscle.

Following a survival period of 21 weeks after the sciatic operation, we investigated the distribution of type I fibres in five hindlimb muscles (ED, FD, GM, PE, TA). Using our methods for the quantification of fibre type regionalization, we mapped out the direction of type I fibre eccentricity within muscle cross-sections taken at different proximo-distal levels. Although the variability was greater than normal, the mean direction of vector regionalization was, for each muscle species, nearly identical to that found in control muscles. Furthermore, the average decline of type I fibre density from proximal toward distal was also similar between reinnervated muscles and controls. In all the reinnervated muscles, but not in the controls, type I fibres were markedly clumped together (extensive “fibre type grouping”), i.e. many of the type II fibres must have been re-specified into type I fibres following the reinnervation. Thus, the similarities in the direction of type I fibre regionalization between reinnervated muscles and
controls were not simply due to the persistence of fibre type distributions present prior to the denervation and reinnervation. These experiments strongly indicate that molecular mechanisms for the “correct” guidance of ingrowing “slow” motor axons are still functional in adult rats.

In Chapter 7 we studied to what extent the recovery of normal patterns of regionalization was dependent on the conditions under which the reinnervation took place. These experiments were all performed on gastrocnemius medialis (GM), a muscle which is normally very clearly regionalized and whose nerve is relatively easily accessible. In all these animals, an initial operation took place under general anaesthesia during which the GM nerve of one hindlimb was transected close to the muscle and then reunited with the same muscle. The succeeding reinnervation-period lasted 21 weeks (cf. Chapter 6) and was followed by a final experiment during which the GM muscle was removed from both hindlimbs and studied using our standard methods. Five groups of animals were compared: (1-2) the cut GM nerve had been re-inserted close to the original nerve entry (group ox) or at a different, more medial site (group dx); (3-4) like groups ox and dx, but also including a rotation of the GM muscle around its longitudinal axis (groups Tox and Tdx); (5) a rotation of the GM muscle like in groups Tox and Tdx, but without cutting the nerve (group Tc). In all the reinnervated muscles (ox, dx, Tox, Tdx), but not in those only rotated (Tc), a marked increase was found of the type I fibre grouping; thus, after reinnervation many of the original type II fibres had become converted into type I. On average, the direction of the type I fibre regionalization was very similar in groups ox and dx and in their contralateral controls. Hence, the guidance of ingrowing “slow” axons toward “slow” muscle regions was apparently equally effective irrespectively of whether the nerve fibres were allowed to regenerate along the original nerve path or were forced to follow another intra-muscular trajectory. In the groups with reinnervation plus muscle rotation (Tox, Tdx) the direction of regionalization was not completely explainable as a result of the muscle rotation itself plus an unchanged pattern of intra-muscular regionalization. These results suggested that the regionalization-associated guidance of ingrowing axons depended on topographic relationships within the muscle as well as within the hindlimb as a whole.

In comparison to the effects of reinnervation along a “foreign” path (dx, Tdx), nerve ingrowth following the original nerve (ox, Tox) was associated with less muscle atrophy, more normal numbers of type I fibres, and less extreme (but still very much increased) levels of type I fibre grouping. As compared to group ox, the reinnervated muscles of group dx had a significantly smaller number of type I fibres. It is still unclear whether this reflected a decrease of the number of surviving “slow” motoneurones and/or a decrease in size of the reinnervated “slow” motor units.

In general, our experiments underline that, among mammalian hindlimb muscles, fibre type regionalization is a very general phenomenon with consistent and well organized features. Our findings provide a starting point for subsequent
enquiries into the functional advantages of this organization. Furthermore, this intramuscular organization of fibre types offers an interesting model for the further analysis of mechanisms for axonal guidance. Finally, our results provide an extensive “map” of hindlimb regionalization features that may be of practical importance in contexts of other muscle research (e.g. in association with electromyographic recordings, muscle biopsies, etc.).