**Minireview**

# Alternative Strategies in Muscle Genotype and Phenotype Studies. A Model of Intrafusal Muscle Fibre Type Differentiation

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**Abstract.** The development and regeneration of muscle fibres start from myoblasts of embryos or adult animals The resulting phenotype is a combination of genetically fixed properties of myoblast cell lineages and of extrinsic, primarily neurogenic factors Intrafusal fibre types of muscle spindles differ from each other and from extrafusal fibres by their ultrastructure, by the presence of both sensory and motor innervation, and by the content of specific myosin heavy chain (MHC) isoforms Differentiation of these distinctions depends on the morphogenetic influence of primary afferent neurones It is however not known whether the intrafusal phenotype can be induced in any myotube regardless of its cell line origin or only in a special predetermined intrafusal lineage(s) committed to differentiate into intrafusal muscle fibres The aim of our studies was to define the contribution of intrinsic myogenic properties of muscle cell lineage and extrinsic neurogenic factors by the sensory and the motor innervation on the differentiation of intrafusal phenotypes using ultrastructural analysis and immunocytochemical determination of MHCs under various experimental conditions The presented minireview is based on the results of our previous findings, and preliminary experiments indicate that new important results may be obtained in studies of myogenesis and muscle regeneration

**Key words:** Muscle development — Muscle cell lineages — Neurogenic influences — Muscle differentiation — Muscle regeneration

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## Muscle differentiation and expression of MHCs in extrafusal muscle fibres

Both myogenesis and regeneration in skeletal muscles are characterized by the fusion of replicating mononucleated myoblasts or satellite cells into syncytial myotubes and by their maturation into differentiated muscle fibres. This differentiation involves activation of cell type-specific genes triggering the synthesis of muscle-type specific proteins. Their expression in a given muscle fibre is a result of the combination of intrinsic programs (cell lineages) and extrinsic (e.g. nerve, hormonal and mechanical influences (for review see Miller and Stockdale 1985, Pette and Staron 1990, Gunning and Hardeman 1991, Hoh 1991, Stockdale 1992). In adult rat skeletal muscles, four major myosin heavy chain (MHC) isoforms exist in the extrafusal fibres: slow twitch MHC is present in type 1 fibres, fast A in type 2A fibres, fast X or D in type 2X/2D fibres, and fast B in type 2B fibres. Developing and regenerating muscle fibres express embryonic and neonatal MHC isoforms (Table 1, for review see Hamalainen and Pette 1995). Other MHC isoforms: slow tonic, extraocular, superfast and alpha-cardiac are as a rule not expressed in limb muscles, but they do occur in highly specialized extraocular and masticatory or the tensor tympani muscles and in intrafusal fibres of muscle spindles (Pedrosa et al. 1990, for review see Soukup et al. 1995).

Expression of muscle MHC genes from late foetal to the adult stages has been analyzed in extrafusal musculature at both protein and mRNA levels using biochemical separation techniques or *in situ* hybridization using MHC probes (for

**Table 1.** Expression of MHC isoforms in rat muscle fibres

| Developing muscle fibres | Adult muscle fibres | | | | |
|---|---|---|---|---|---|
| | Extrafusal | | Intrafusal | | |
| | type 2 (fast) | type 1 (slow) | nuclear $bag_1$ | nuclear $bag_2$ | nuclear chain |
| embryonic<br>neonatal | 2A<br>2B | 1 | embryonic<br>slow twitch / beta cardiac | embryonic<br>neonatal | neonatal*<br>fast twitch* |
| | 2X/D | | slow tonic* | slow twitch / beta cardiac* | |
| | | | alpha cardiac | slow tonic<br>alpha cardiac<br>fast twitch | |

*These isoforms are distributed along the whole fibre length (for details see Soukup et al. 1995)

review see Pette and Staron 1990 Gunning and Hardeman 1991 Hoh 1991 Ontell et al 1995) However biochemical or clonal analyses of differentiated intrafusal fibres are difficult due to their scarcity and the corresponding difficulties concerning their isolation (cf Pedrosa-Domellof et al 1993) Such studies of tiny undifferentiated intrafusal fibres in developing muscle spindles are still virtually impossible also due to the general problems in gaining access and manipulating mammalian foetuses We have therefore tried to induce experimentally postnatal myogenesis and regeneration inside rat muscle spindles These experiments would make it possible to analyze the contribution of intrinsic myogenic (cell lineage) and extrinsic (neurogenic) factors on the ultrastructural differentiation and expression of MHC isoforms in intrafusal fibres The anticipated results should fill the gap resulting from the lack of biochemical results in the studies of differentiation of intrafusal muscle fibre types

## Characteristic distinctions of intrafusal muscle fibres

Intrafusal muscle fibre types exhibit distinct morphological characteristics as they contain typical nuclear accumulations and myofibrillar ultrastructure possess complex sensory and motor innervation exhibit specific histo- and immunocytochemical characteristics and are surrounded by a multilayered capsule (Figs 1 2 3 for review see Zelena 1994, Soukup et al 1995) Each intrafusal fibre type expresses a

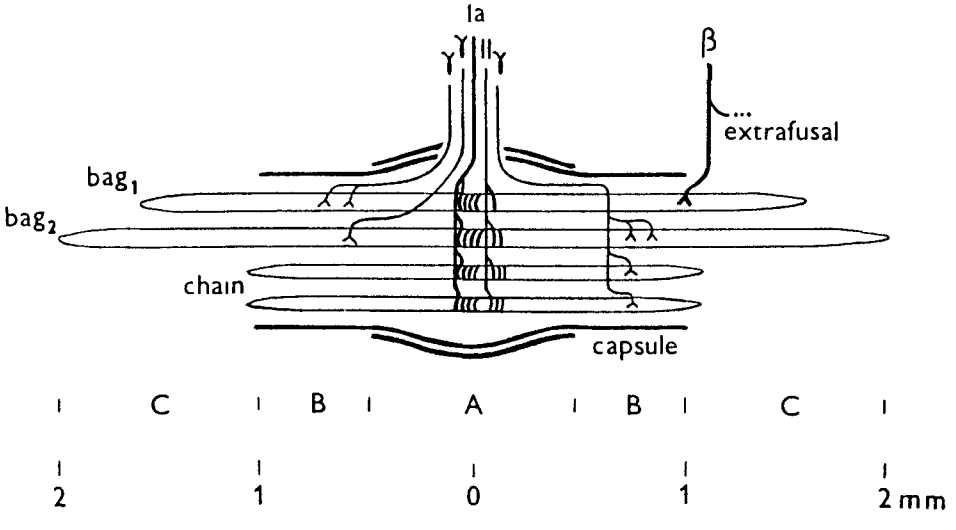


**Figure 1.** Schematic drawing of a rat muscle spindle (not drawn to scale) $bag_1$ $bag_2$ chain intrafusal muscle fibres Ia primary sensory axon II secondary sensory axon $\gamma$ and $\beta$ motor innervation A B,C – the A B and C spindle regions (modified from Pedrosa-Domellof 1991)

typical MHC pattern (Table 1) comprising at least 6 MHC isoforms in nuclear $bag_2$ fibres (embryonic, neonatal, slow twitch/beta cardiac alpha cardiac slow tonic and fast twitch) 4 MHCs in nuclear $bag_1$ fibres (embryonic slow twitch/beta slow tonic and alpha cardiac), and 2 MHCs in nuclear chain fibres (neonatal and fast twitch) The distinct ultrastructure characteristic MHC patterns and expression of spindle-specific MHC isoforms (not expressed in extrafusal fibres) distinguish intrafusal fibre types from each other and from extrafusal fibres (for review see Soukup et al 1995) Especially the "slow tonic" MHC expressed from an early developmental stage onwards is a reliable and unique marker of intrafusal fibres in developing and adult muscle spindles Intrafusal fibres thus represent a naturally occurring model for the analysis of myogenesis and for the study of intrinsic myogenic and extrinsic neurogenic or other factors regulating muscle development and regeneration

The first studies suggested that intrafusal fibres in muscle spindles of newborn and adult rats differ in their reactivity to polyclonal antisera against MHCs (Pierobon-Bormioli et al 1980 te Kronnie et al 1981 1982) Further studies revealed that the unique expression of slow tonic alpha cardiac embryonic and neonatal MHCs in limb muscles of adult mammals is restricted to the intrafusal fibres (Maier et al 1988 Kucera and Walro 1989 1990, Pedrosa et al 1990 Pedrosa-

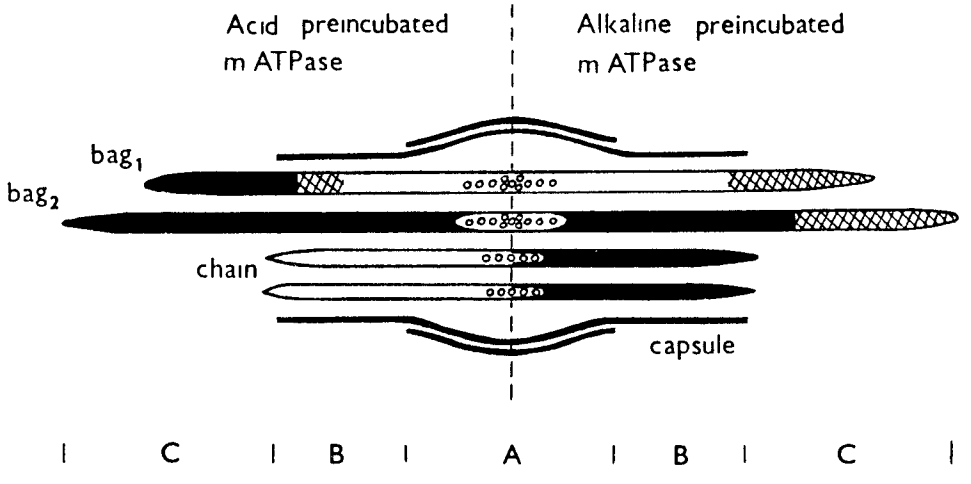


**Figure 2.** Schematic representation (not drawn to scale) of the mATPase activity after acid (pH 4.35) and alkaline (pH 10.3) preincubations along the length of rat intrafusal fibres Extracapsular ends of nuclear bag fibres however exhibit alkali stable activity that varies from low to high levels The circles in the central region of each intrafusal fibre represent nuclei three levels of staining intensity are distinguished black = strong grey = moderate white = weak or unstained (modified from Pedrosa-Domellof 1991)

Domelloff et al 1991, for review see Soukup et al 1995) It was also reported that the reactions with different antibodies against MHCs vary along the length of intrafusal fibres (Pedrosa et al 1990, Soukup et al 1990a, Pedrosa-Domellof et al 1991) and this can explain the regional differences observed earlier in the mATPase reaction (Fig 2, Soukup 1976) Various studies have also shown that sensory innervation is required for the expression of spindle-specific MHC isoforms and that motor innervation contributes to the diversity in the distribution of the different MHCs along the length of the nuclear bag fibres (Kucera and Walro 1988 Soukup et al 1990a, for review see Zelená 1994, Soukup et al 1995)

The major question pertaining to spindle development is whether intrafusal fibres develop from the same pool of bipotential muscle precursor cells (myoblasts) as extrafusal fibres or from a unique population(s) of myoblasts predestined to become intrafusal fibres Primary myotubes, which give rise to the first generation of both extrafusal and intrafusal fibres, do not differ in their ultrastructure at the onset of spindle development Hitherto, no difference has been detected in their immunocytochemical profiles either, before they are contacted by sensory axons

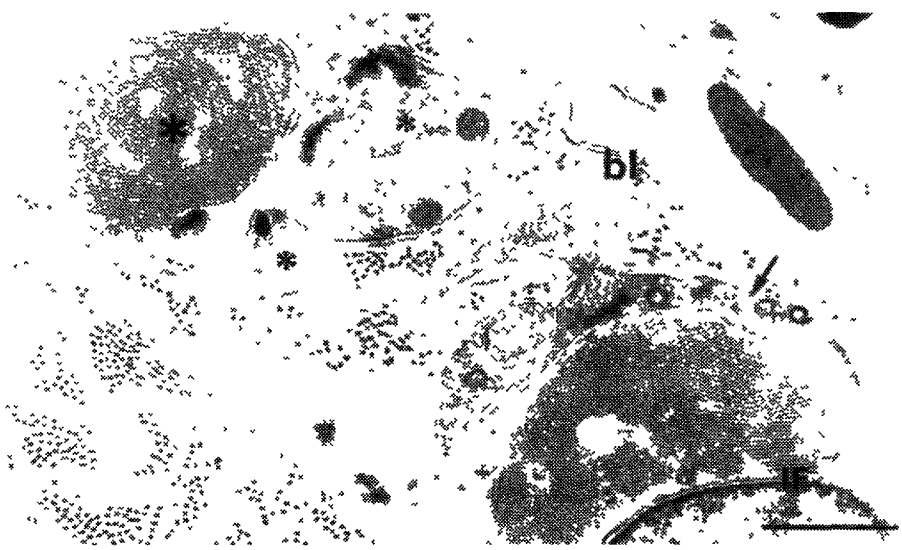


**Figure 3.** A newly formed intrafusal fibre (large asterisk) and two immature intrafusal myotubes (small asterisks) probably originated from an adjacent parent intrafusal fibre (IF) from a spindle of a four-month-old rat deefferented 48 h after birth A presumably activated sickle-shaped myoblast (arrow) is located on the fibre surface and covered by the basal lamina (bl) of the intrafusal fibre The scale bar represents 1 $\mu$m (× 20 000)

## Alternative experiments for studies of intrafusal muscle fibre type origin

*Deafferentation and denervation experiments* We have found that neonatal deafferentation performed during the early postnatal period by the extirpation of the lumbosacral spinal cord triggered neomyogenesis which led to a gradual increase in the number of intrafusal fibres (Figs 3 and 6) and that new fibres were formed predominantly from activated satellite cells (Soukup et al 1993, Zelena and Soukup 1993) Our ultrastructural (Fig 4 Novotova and Soukup 1995) and preliminary immunohistochemical results in young rats (Soukup et al 1994a b) showed that neomyogenesis can also be achieved directly by denervation of muscle spindles or by deafferentation followed by denervation if nerve section is performed after the critical period of muscle spindle development, i e when muscle spindles survive the loss of sensory innervation (for review see Zelena 1994 Soukup et al 1995)

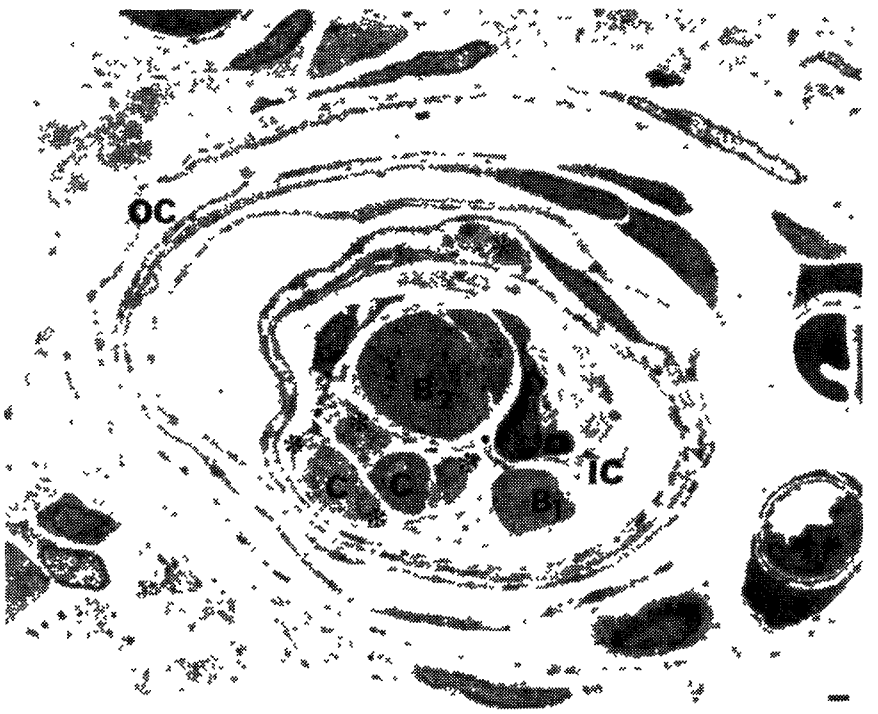


**Figure 4** A transverse section of a muscle spindle from a 2-month-old rat deafferented at 2 days and denervated at 4 weeks The spindle contains 8 large profiles at this level presumably corresponding to nuclear bag2 (B2) nuclear bag1 (B1) and nuclear chain (C) fibres and to the *de novo* formed supernumerary intrafusal profiles (asterisks) OC outer capsule IC inner capsule The scale bar represents 1 $\mu$m (× 2 900)

These models represent a unique possibility to study fibre type differentiation from mononucleated adult myoblasts (satellite cells) of distinct types given and defined by their spatial relation to differentiated parent intrafusal fibres and the postnatal stage of the experimental animals enables easier anatomical access for surgical manipulations

*Muscle grafting experiments* The sequence of events during muscle regeneration after muscle transplantation is similar to muscle development (for review see Carlson 1976) An alternative model of examining muscle postnatal neomyogenesis and differentiation of intrafusal fibres during the postnatal period is thus regeneration following muscle grafting (Fig 7) The detailed description of the method of heterochronous allotransplantation and determination of muscle spindle numbers in the regenerated grafts has already been published (Jirmanová and Soukup 1995) Muscle regeneration can thus be used as another experimental model for the studies of genetic and neural influences on the differentiation of phenotypes of intrafusal muscle fibres

In order to elucidate the role of myogenic predetermination versus plasticity of adult myoblasts and the manner in which innervation contributes to the development of the regional heterogeneity the ultrastructural differentiation and the MHC expression can be studied by transmission electron microscopy and immunocytochemical detection of MHC isoforms using a battery of specific monoclonal antibodies under various experimental conditions In our studies, we have focused on a) neonatally deefferented spindles postnatally deprived of sensory innervation by nerve section, b) reinnervated atypical spindles after neonatal nerve crush c) denervated muscle spindles in young and adult rats and d) muscle spindles regenerating after heterochronous allotransplantation and autotransplantation of whole hind limb muscles in the presence or absence of either sensory or motor innervation

*Neonatal deefferentation* was performed by the extirpation of the lumbosacral spinal cord in 6 to 48-hour-old rats under cold anaesthesia (for details see Zelena and Soukup 1993 Soukup et al 1993)

*Neonatal nerve crush* of the sciatic nerve was done under cold anaesthesia by a pair of forceps at the mid-thigh level in 6-hour-old rats (for details see Soukup and Zelena 1988)

*Postnatal denervation* was made by the section of the sciatic nerve at the mid-thigh level in 3- to 4-week-old and adult rats under Nembutal anaesthesia (for details see Novotova and Soukup 1995)

*Heterochronous allotransplantation* was performed by grafting, e g the extensor digitorum longus muscle from 2- to 28-day-old rats into the same muscle of adult recipient inbred rats using cold and Nembutal anaesthesia (for details see Jirmanova and Soukup 1995)

*Autotransplantation* was carried out in 2-month-old rats under Nembutal anaesthesia, the right extensor digitorum longus muscle was excised, its nerve and blood

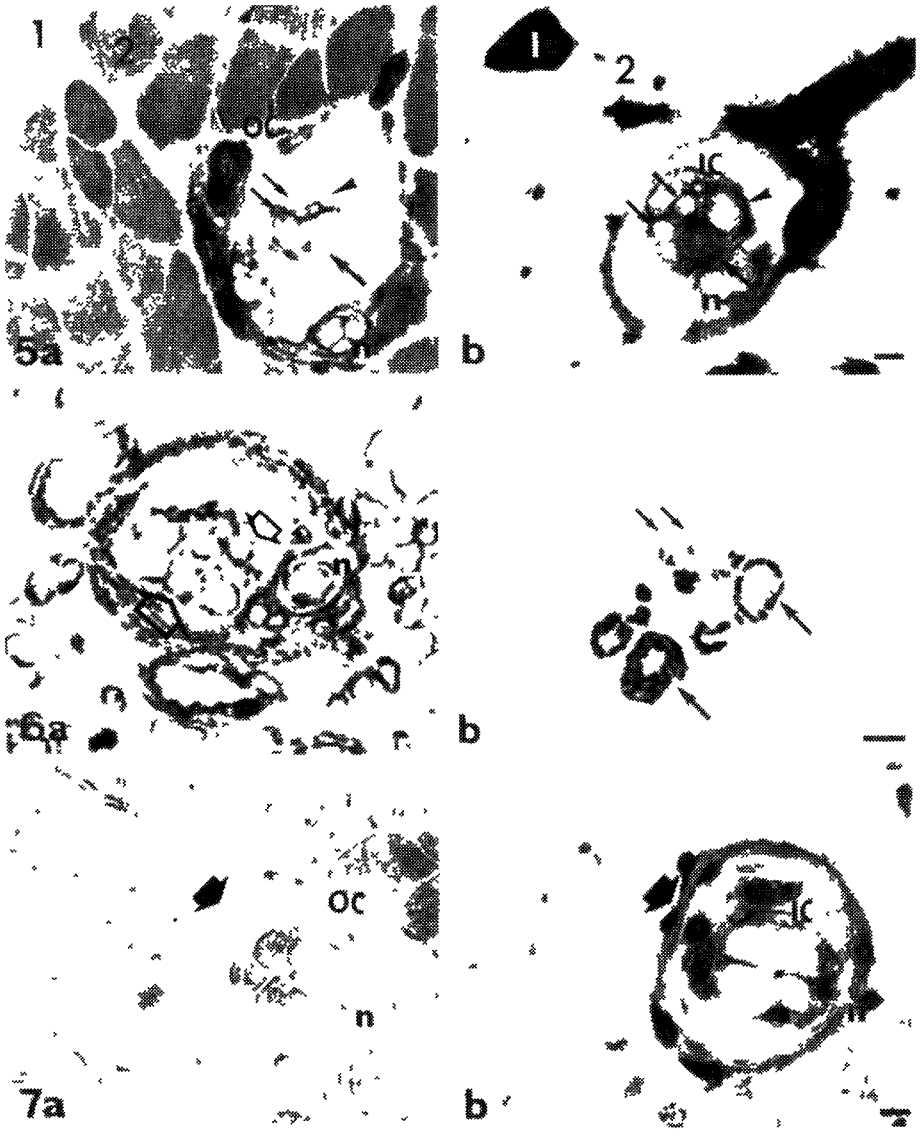


**Figure 5.** Serial transverse sections through the A region of a normal spindle from an EDL muscle of an adult rat stained for m ATPase activity after preincubation at pH 10 3 (*a*) and 4 35 (*b*) In the control, intrafusal fibres are differentiated into three fibres types – nuclear bag$_2$ fibres (large arrow) nuclear bag$_1$ fibres (arrowhead) and nuclear chain fibres (small arrows) 1 extrafusal type 1 fibres, 2 extrafusal type 2 fibres OC outer capsule IC inner capsule n – spindle nerves The bar represents 10 $\mu$m (× 400)

**Figure 6.** Cross sections through the A zones of two deefferented spindles from a soleus muscle of a 6-week-old rat stained with monoclonal antibodies against laminin (a) and slow

supply was cut and the muscle tendons were sutured back to their original site (for details see Carlson 1976)

## Results and future prospects

The result of our studies of MHC expression in neonatally deefferented rat muscle spindles (te Kronnie et al 1982 Pedrosa et al 1990 Soukup et al 1990a Pedrosa-Domellof et al 1991 Soukup et al 1993, for review see Soukup et al 1995) led us to the assumption that there are at least two types of intrafusal satellite cells (adult myoblasts) namely those derived from slow nuclear bag and those related to fast nuclear chain fibres (Fig 5) This assumption is in agreement with the previous suggestion that intrafusal fibres originate from a unique population(s) of myoblasts destined to become intrafusal fibres (Pedrosa and Thornell 1990 Soukup et al 1993 1995) although it is at variance with the suggestion that intrafusal fibre types develop from the same populations as extrafusal fibres (Kucera and Walro 1990 Zelena 1994)

The obtained and expected results prompt us to ask the following questions

*1) What is the specific genetic determination of intrafusal adult myoblasts?*

Experiments test whether the adult myoblasts (satellite cells) are predetermined and differentiate into specific phenotypes of intrafusal fibre types in the absence of their innervation We already know that satellite cells differentiate into parent fibre types in the absence of motor innervation (Figs 5 and 6 Soukup et al 1993 Zelena and Soukup 1993) Preliminary experiments have shown that also *de novo* formed denervated intrafusal fibres can express the spindle-specific slow tonic like MHC if they develop in spatial relation to nuclear bag fibres but not if they had differentiated from the satellite cells of nuclear chain origin (Soukup et al 1996) Conclusions from these experiments can contribute to the verification of the proposed hypotheses regarding the origin of intrafusal fibres

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tonic MHC (*b*) Note the increased number of intrafusal fibres with large (open arrow) and small (small open arrow) diameters in (*a*) and the presence of positively stained intrafusal profiles of various size (large arrows) and small unstained fibres (small arrows) in (*b*) The stained fibres are of the nuclear bag type whereas the unstained fibres belong to the nuclear chain type fibres The bar represents 10 $\mu$m (× 620)

**Figure 7.** Serial transverse sections through the A zone of a regenerated spindle (full arrow) from an EDL muscle taken for allotransplantation from a 28-day-old rat 1 month after grafting into the adult inbred recipient rat Sections were stained for m-ATPase after alkaline (*a*) and acid (*b*) preincubations Regenerated encapsulated fibres are not differentiated into three intrafusal fibre types typical for the control spindles but they resemble extrafusal type 2 fibres (2) For other descriptions see legend to Fig 5 (× 100)

*2) How much are adult intrafusal myoblasts predetermined or plastic?*

Experiments analyse how foreign (extrafusal) motor innervation can modify the differentiation of regenerating intrafusal fibres (Figs 5 and 7). We have already shown that mATPase reaction of regenerated intrafusal fibres after allo- and autotransplantation resembles that of extrafusal type 1 or type 2A and 2B fibres (Soukup 1988; Jirmanova and Soukup 1995) and that they fail to express the spindle-specific ultrastructural distinctions and slow tonic and alpha cardiac-like MHC isoforms (Soukup et al. 1990b; Soukup and Novotova 1996). These results show that both types of intrafusal satellite cells derived from either nuclear bag or nuclear chain fibres exhibit considerable plasticity as their MHC expression can be respecified towards extrafusal muscle phenotype by foreign motor innervation. The conclusions from these experiments indicate under which conditions intrafusal satellite cells retain their ability to differentiate into intrafusal or extrafusal fibre types.

*3) What determines the regional heterogeneity of MHC expression?*

Experiments can provide the answer to the question of whether and how the differences in ultrastructure and in expression of MHC isoforms along the length of intrafusal fibres are related to the influence of sensory and motor innervation and to the properties of the myoblast lineages. It has already been shown that a number of proteins including MHC isoforms can remain localized in the vicinity of the nuclei responsible for their synthesis (for review see Hall and Ralston 1989; Pavlath et al. 1989). The concept of nuclear domains can thus be applied to the intrafusal fibres as the typical regional variations along their length might reflect the existence of nuclear domains under the influence of either sensory or motor innervation (for review see Soukup et al. 1995).

## Conclusions

The proposed strategy not only can fill the gap resulting from the lack of biochemical results in studies of differentiation and regeneration of intrafusal muscle fibre types, but it also can provide valuable results since recognition of factors inducing differentiation of immature muscle precursors into individual adult types is of basic importance for developmental neurobiology and since the knowledge of factors operating in the process of muscle spindle development and regeneration has implications for neurology, neurosurgery and plastic surgery.

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