

Nerve Influence on Rat Fast-Twitch Skeletal Muscle Regeneration

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Abstract

The degeneration-regeneration process induced by bupivacaine injection has been studied in rat extensor digitorum longus (EDL) muscle in the presence or absence of nerve, the presence of tetrodotoxin (TTX)-induced block of nerve impulse conduction, and the presence of vinblastine-induced block of nerve axoplasmic flow associated to TTX block. Seven and 14 days after bupivacaine injection, myosin heavy chain (MHC) expression was analysed by immunohistochemistry and Western blot. Type 1 MHC isoform expression was almost completely dependent on nerve impulse discharge. Expression of the type 2A showed a zonal distribution in denervated muscles, and a marked down-regulation in the TTX-paralysed, but not in TTX-vinblastine-treated muscles. These results show that expression of type 2A isoform in innervated-regenerated fast muscle is mainly due to neuromotor activity. They also suggest that 2A isoform expression is inhibited by a chemical factor carried by axoplasmic flow.

Key words: fast skeletal muscle, myosin heavy chains, nerve activity block, neurotrophic factors, regeneration.

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The basic role played by innervation for the correct differentiation of slow muscle regenerating after acute degeneration is well documented (for references see [13]). It has recently been shown that in regenerating rat soleus muscle, the expression of type 1 and type 2A myosin heavy chain (MHC) isoforms is strictly dependent on neuromotor impulses [12, 13] since these isoforms are not present when muscle regenerates in the presence of impulse conduction block in the sciatic nerve. However, after surgical denervation, as well as in the presence of axoplasmic flow blockade of sciatic nerve, regeneration is accompanied by a diffuse expression of type 2A isoform, suggesting that the nerve also exerts a chemical, inhibitory influence on the expression of this isoform [12].

There is relatively little information available regarding the role of innervation in fast muscle regeneration. Fast and slow muscles have a different developmental history [9, 10, 16] and may have a different nerve-dependence for differentiation [9, 16]. Indeed, expression of fast MHC isoforms during muscle development appears to be less affected by denervation than expression of slow isoform [9, 16]. Muscle regeneration is similar to prenatal myogenesis and it could be expected that during regeneration, fast muscle is also less dependent on innervation for its differentiation. Esser et al. [7] found that the acquisition of a fast mRNA profile by regenerating EDL muscle does not require innervation and as reported by both Cerny and Bandman [5], and d'Albis et al. [6], fast type

MHC isoform expression is also independent of innervation. However, in some regenerating muscle, innervation appears to be necessary for the formation of normal adult fibres and for the repression of neonatal MHC [5], while denervation favours the synthesis of the type 2A isomyosin at the expense of fast type 2B isoform [6].

The aim of the present study was to extend to the fast muscle the investigation on the role of innervation in type 1 and 2A myosin expression during regeneration. In the rat EDL muscle, we compared regeneration in innervated muscles with that in surgically or functionally denervated muscles. Functional denervation was obtained by blocking impulse conduction in the sciatic nerve. The effect of vinblastine-induced axoplasmic flow interruption in the nerve was also investigated.

Materials and Methods

Fifty adult Wistar rats, 250-300 g in weight, were used and assigned randomly to the different experimental groups. All the experiments were carried out in accordance with the Helsinki Accords for Human Treatment of Animals during Experimentation. The study was approved by the Ethics Committee of the Medical Faculty of the University of Padova.

Surgical procedures

Degeneration/regeneration was induced in the EDL muscle of both hind limbs by injection of 0.5-0.7 ml of 0.5% bupivacaine solution [1]. Muscles were accessed

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through a small cutaneous incision. All surgical procedures were performed in a single session under general ether anaesthesia. Four groups of muscles were utilized: i) muscles regenerated in the presence of normal innervation; ii) muscles regenerated after the section of the ipsilateral sciatic nerve at level of the trochanter (1.5 cm of the peripheral nerve stump was removed to avoid reinnervation); iii) muscles regenerated in the presence of chronic block of sciatic nerve impulse conduction; iv) muscle regenerated in the presence of both impulse conduction and axoplasmic flow block. The nerve impulse conduction block was obtained by superfusion with tetrodotoxin (TTX, Sankyo, Japan, 500 µg/ml), released by a mini-osmotic pump (Alzet 2002, 200 µl volume, 0.5 µl/hr release rate, 14 days discharge time) [2]. The pump was implanted subcutaneously in the interscapular region and connected by means of a polyethylene catheter to a silicon cuff surrounding the sciatic nerve [12, 13]. Axoplasmic flow was blocked by applying cotton wool soaked with 0.15 mM vinblastine (Sigma, USA) in 0.9% saline around the nerve for 20 min, according to the technique described by Kashiba et al. [11]. This procedure ensures an axonal flow blockade without neuronal damage [11, 12, 20, 21]. Seven or fourteen days after bupivacaine injection, muscles were excised and immediately frozen in liquid nitrogen for subsequent analysis.

Fibrillation recording

Electromyographic recordings were made under general ether anaesthesia from the hind limb muscles using a pair of needle electrodes (Beckman, Fullerton, California), insulated except for the tips, with an interelectrode distance of 2 mm and inserted transcutaneously. Bioelectric signals were amplified by a differential amplifier (5A22N, Tektronix, Beaverton, Oregon), using a bandwidth of 1-3 kHz, and displayed on the screen of a cathode-ray oscilloscope (5103N, Tektronix, Beaverton, Oregon). Fibrillation activity was recorded 7 and/or 14 days after bupivacaine injection.

Control of nerve block and nerve integrity

The patency of the nerve block was checked daily by monitoring the presence of paralysis of the leg and the absence of withdrawal and toe-spreading reflexes in the experiments with TTX [14]. The absence of paralysis was also checked in the experiments with vinblastine. Before sacrificing the animals, the nerve block was tested by directly stimulating the nerve as previously described [12, 13]. The nerve was considered blocked if the stimulation above the cuff surrounding the nerve, connected to the miniosmotic pump used to release TTX, did not elicit any limb movement, whereas stimulation below the cuff elicited a visible response.

Immunohistochemical analysis

Serial cross sections (8 µm thick) were cut in a cryostat microtome at $-23 \pm 2^\circ\text{C}$ (Slee Pearson, England). The immunofluorescence analysis was performed on the serial

sections by using primary monoclonal antibodies (mAbs) (generous gift of Prof. S. Schiaffino) specific against type 1 (BA-D5), type 2A (SC-71) and embryonic MHC (BF-G6) [8, 15]. A TRITC-conjugated rabbit anti-mouse immunoglobulin (Dako, Denmark) was used as the secondary antibody. We identified and counted fibres on enlarged microphotographs acquired by Leica DC100.

Immuno-electrophoretical analysis

Identification of MHC isoforms, was performed by the method described by Talmadge and Roy [18]. Twenty-five serial cryosections (20 µm thick) from the muscle regenerated portion were collected in an eppendorf tube, weighted and dissolved at a concentration of 2 mg/ml in SDS-PAGE solubilization buffer. Particular care was taken to use the regenerated portion of the muscle. The persistence of unaffected portions was preliminarily verified by staining with haematoxylin-eosin. The regenerated areas were distinguished by the presence of smaller fibres with central nuclei. The unaffected portion of the muscle was removed with a razor blade to avoid contamination [13]. Forty µg of each sample were separated by electrophoresis on 8% SDS-PAGE slab gels and successively transferred on nitrocellulose filter by Western blot. The nitrocellulose filter was incubated for 1 h with the monoclonal antibodies specific for 1 or 2A MHC. Then the filter was incubated with the secondary antibody (peroxidase conjugated goat anti mouse immunoglobulins, Dako, Denmark, 1: 2000) for 1 h. The immunocomplexes were visualized by diaminobenzidine staining. The relative amount of each myosin heavy chain isoform was determined by densitometry of immunolabelled protein bands on nitrocellulose filters using a Bio-Rad Imaging Densitometer (GS-670).

Statistical analysis

Means $\pm$ SE were calculated and were analysed with Student's *t*-test for unpaired data.

Results

A single intramuscular injection of bupivacaine solution caused degeneration in about half muscle. In most cases, regenerated portions of the muscle were intermingled with pre-existing, undamaged portions, making it very difficult to obtain uncontaminated preparations suitable for analysis by electrophoresis (see below).

Fibrillation activity

Fibrillation was present in all denervated muscles. It was instead usually undetectable in TTX-, and in TTX-vinblastine-blocked muscle preparations. To avoid the possible effects of fibrillation on MHC expression [13], the nerve-blocked preparations showing spontaneous activity were not used in subsequent analyses.

Immunofluorescence analysis

Under all experimental conditions, the fibres reacted intensively at 7 days and only slightly at 14 days with the anti-embryonic MHC mAb (not shown).

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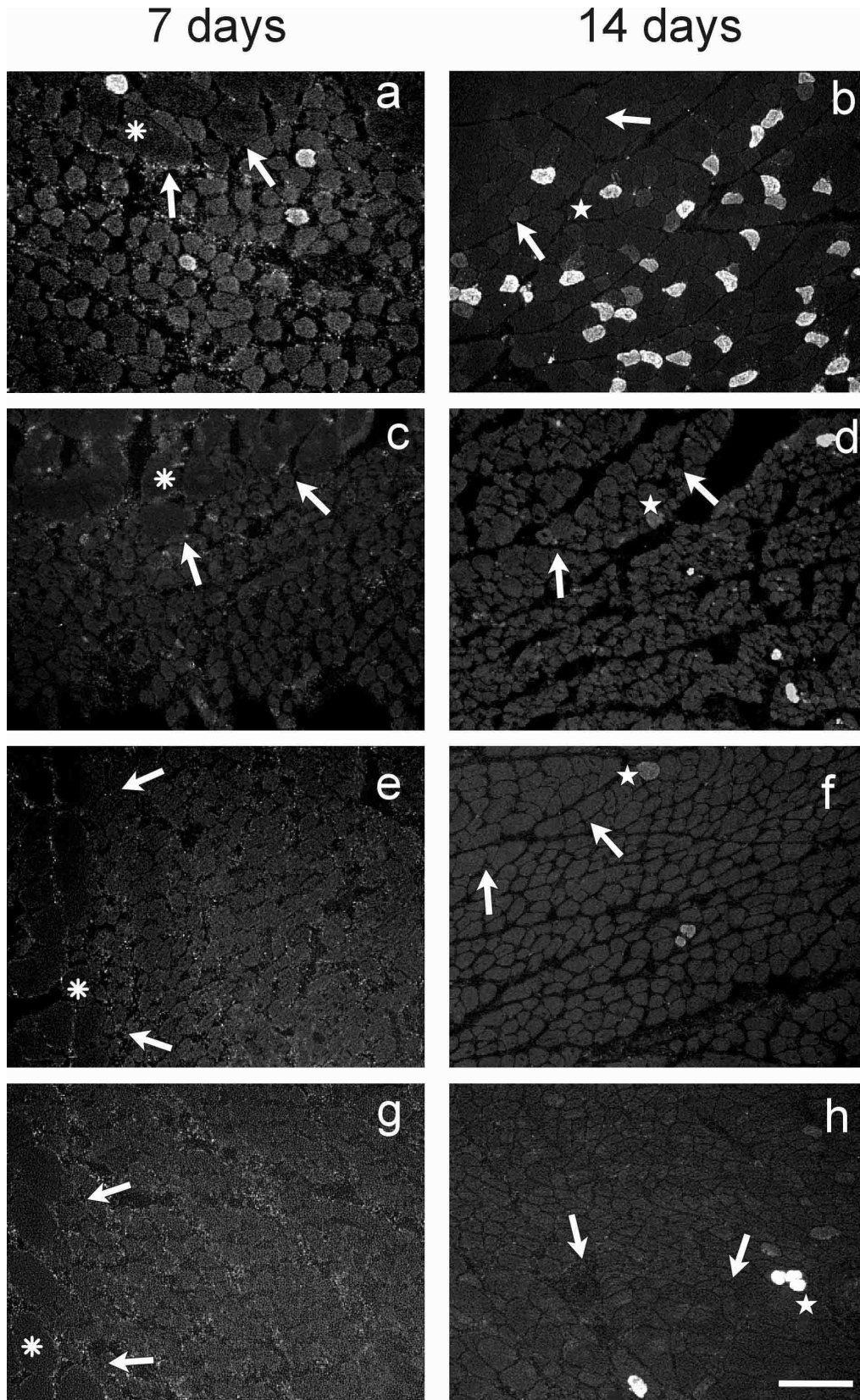


Figure 1 Immunofluorescence staining of sections from 7 and 14 day regenerated EDL muscles with monoclonal antibody against the type 1 MHC isoform. Innervated (panels a, b), denervated (panels c, d), TTX-blocked (panels e, f), and TTX-vinblastine-blocked (panels g, h) muscles. Arrows indicate a pre-existing, undamaged region. (Scale bar = 100 μ m). The asterisks and the stars indicate the same fibre in serial sections, at 7 and 14 days respectively.

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Innervated muscle

Seven days after bupivacaine treatment (8 muscles analysed), rare fibres reactive with anti-1 mAb were found in 7 out of 8 muscles (Fig. 1, panel a). The majority of fibres were only slightly reactive with the anti-2A mAb, while a few fibres stained more intensively (Fig. 2, panel a).

After fourteen days (6 muscles analysed), numerous fibres containing type 1 MHC were found (Fig. 1, panel b), the majority of which also contained type 2A MHC. Most fibres were more markedly labelled by anti-2A mAb than that seen at 7 days (Fig. 2, panel b).

Compared to the values obtained from the unaffected portions of the same muscles, the percentage of regenerated fibres expressing type 1 or type 2A MHC isoforms was higher (type 1: 21.2 ± 6.1 vs. 5.3 ± 2 ($P < 0.05$), and type 2A: 43.4 ± 6.9 vs. 32.2 ± 6.9 ; data from 4 muscles). A great percentage of fibres (28.7 %) expressed both isoforms, while fibres expressing only type 1 or type 2A were about 12% and 60%, respectively.

Denervated muscles

Seven days after bupivacaine injection (6 muscles analysed), no fibres reacting with the anti-1 MHC mAb were found (Fig. 1, panel c). All fibres were slightly reactive with the anti-2A (Fig. 2, panel c).

Fourteen days after bupivacaine injection (9 muscles analysed) generally no fibres were labelled with anti-1 MHC mAb (Fig. 1, panel d); however, in some cases isolated fibres containing type 1 MHC were found. The reaction with the anti-2A MHC antibody (Fig. 2, panel d) showed a patchy distribution: some portions of the muscle were rich in fibres showing an intense reaction with the antibody, whereas other areas were unreactive or showed weak labelling in sparse fibres (Fig. 3, panels a, b).

TTX-treated muscles

Seven days after bupivacaine injection (3 muscles analysed), no fibres reacting with the anti-1 MHC mAb were found (Fig. 1, panel e), while almost all fibres were slightly labelled by anti-2A mAb. Intensively stained fibres were rare (Fig. 2, panel e).

At 14 days (7 muscles analysed), no type 1 fibres were present (Fig. 1, panel f), except in one case where only rare fibres were found. Some fibres were slightly positive for the type 2A isoform (Fig. 2, panel f).

TTX-vinblastine-treated muscles

At 7 days (3 muscles analysed), TTX-vinblastine-treated muscles appeared similar to muscles denervated for seven days. No fibres containing the type 1 MHC isoform were present (Fig. 1, panel g). All fibres were slightly reactive with the anti-2A (Fig. 2, panel g).

Fourteen days after bupivacaine injection (3 muscles analysed), rare fibres expressing type 1 MHC were present (Fig. 1, panel h) and several fibres (more than in only TTX-treated muscle) contained type 2A MHC isoform (Fig. 2, panel h). Type 2A fibres were well differ-

entiated and showed a patchy distribution similar to that seen in denervated-regenerated muscles.

Western blot analysis

Because of the difficulty of obtaining regenerated muscle preparations uncontaminated by adult non degenerated fibres, Western blot analysis was limited to a single case for each experimental conditions, and the results must be considered as representative examples of the changes in the amount of MHC isoforms (Table 1). These results are consistent with those obtained with immunohistochemical analysis in that they show an increase of type 1 MHC isoform in the regenerated innervated muscle, and the absence of this isoform both in regenerated denervated and regenerated TTX-paralysed preparations. Moreover, they confirm the low expression of type 2A MHC isoform in the regenerated TTX, and the higher expression in regenerated TTX-vinblastine-treated muscles.

Discussion

Our results confirm that fast muscle is more resistant to anaesthetics induced degeneration than slow muscle [1]. In fact, in EDL muscle the injection of bupivacaine caused a less massive degeneration than in soleus muscle [12], with a parcelled distribution of the degeneration process. Our results with 14-day regeneration experiments show that the expression of type 1 MHC isoform is almost completely dependent on nerve impulse discharge, while the type 2A MHC isoform is partially present also in absence of neuromotor impulses.

At seven days appreciable differences were noticed in the expression of the slow MHC isoform under the different experimental conditions. In fact, the type 1 isoform was present in nearly all innervated-regenerated muscles and absent in denervated- and in TTX-paralysed muscles. It seems reasonable to attribute this difference to the resumption of neuromotor activity in regenerated muscle. We have not evaluated the time course of reinnervation in regenerated muscles, but in other models of muscle regeneration the first nerve-muscle contacts were shown to occur on the fourth day (for references see [17]) with full innervation occurring by seven days [19].

At 14 days the type 1 isoform was expressed by very rare fibres in denervated as well as in TTX-paralysed muscles, while in innervated muscles the expression was up-regulated with respect to the control muscles. The

Table 1. MHC isoform composition in regenerated EDL.

	type 1 MHC	type 2A MHC
control	4.9 %	14.9%
regenerated innervated	17.6%	44.0 %
regenerated denervated	0	18.6%
regenerated TTX-treated nerve	0	10.6 %
regenerated TTX-vinblastine-treated nerve	1.4 %	24.9 %

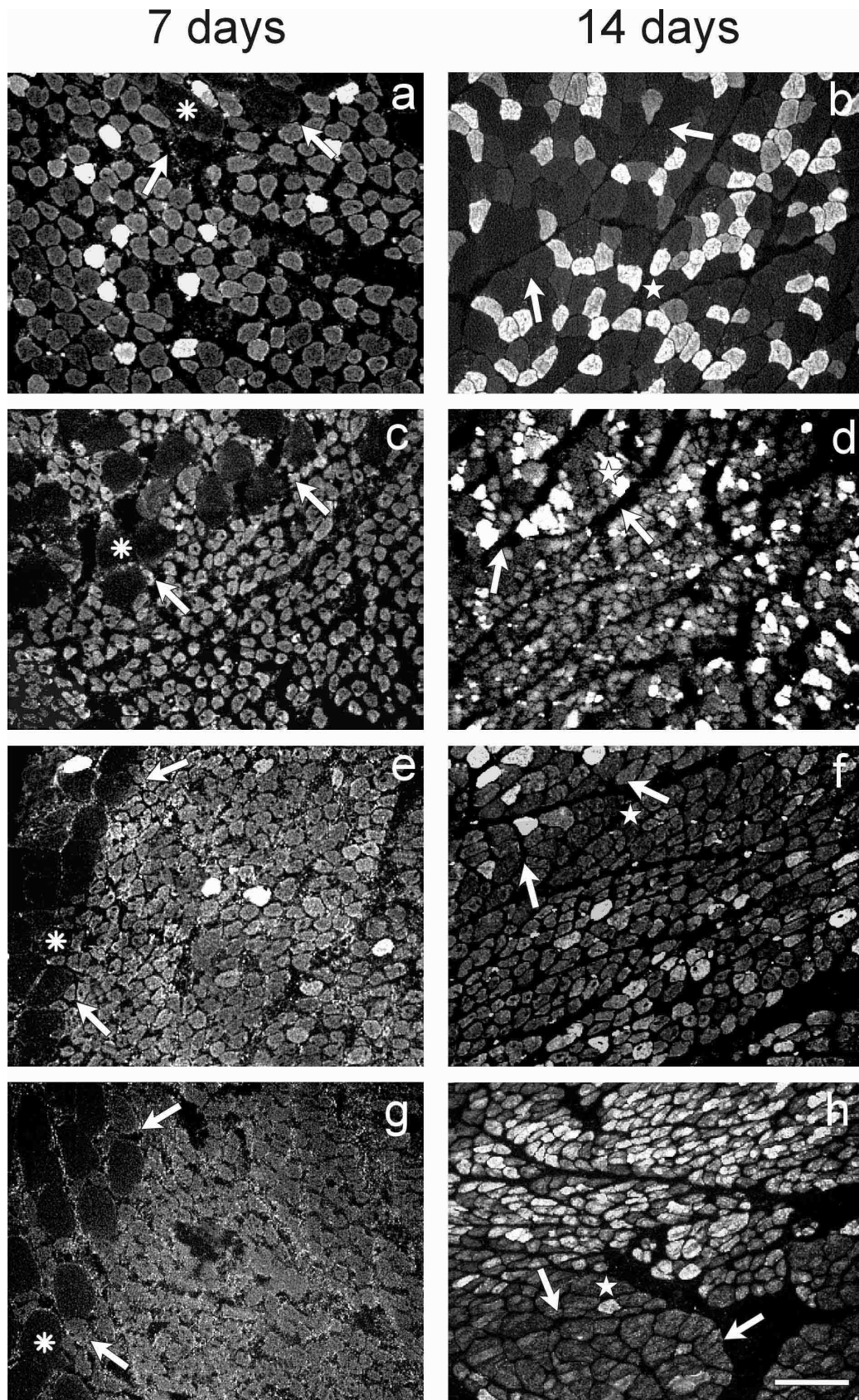


Figure 2 Immunofluorescence staining of sections from 7 and 14 day regenerated EDL muscles with monoclonal antibody against the type 2A MHC isoform. Innervated (panels a, b), denervated (panels c, d), TTX-blocked (panels e, f), and TTX-vinblastine-blocked (panels g, h) muscles. Arrows indicate a pre-existing, undamaged region. (Scale bar = 100 μ m). The asterisks and the stars indicate the same fibre in serial sections, at 7 and 14 days respectively.

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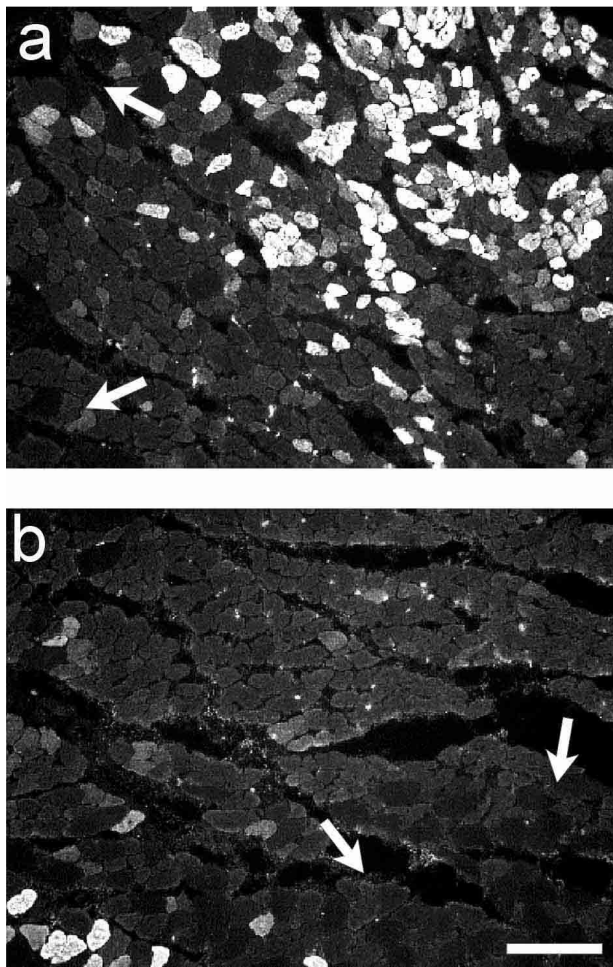


Figure 3. Immunofluorescence staining with anti-2A MHC mAb of different portions of the same section from 14-day denervated-regenerated EDL muscle. Note the high level in panel a, and the absence of expression of 2A MHC isoform in panel b. Arrows indicate a pre-existing, undamaged region. (Scale bar = 100 μ m).

presence in denervated or TTX-paralysed muscles of fibres expressing type 1 isoform – which was not observed under the same experimental conditions in regenerated soleus muscle (13) – suggests that in regenerating fast muscle is present a cell lineage that can differentiate into type 1 fibres independently of innervation, similar to primary generation myoblasts [16]. The up-regulation of the type 1 isoform following regeneration is known to occur in slow muscle [17, 19]. In the EDL muscle of sedentary animals [3], a slight increase in the number of fibres expressing the type 1 isoform has been observed at 5 weeks after induction of muscle degeneration, but no differences were reported after 10 weeks [4]. We can only note that the up-regulation of type 1 fibres occurred in innervated but was absent in TTX-treated muscles, and this forces us to conclude that it was strictly dependent on neuromotor discharge to the muscle.

The differences in type 2A MHC expression observed at 14 days are of particular interest. In innervated-regenerated muscle, type 2A MHC fibres were rather homogeneously distributed in the muscle. In the denervated-regenerated muscle, they showed a zonal distribution with portions of regenerated muscle that were completely unreactive with the anti-2A mAb. This observation indicates that in regenerating EDL muscle only a portion of muscle fibres is programmed to express type 2A MHC isoform and that the normal distribution of positive fibres is dependent on innervation. The results with the TTX nerve-block demonstrate the prominent role played by neuromotor impulses in inducing expression of this isoform. Indeed, in TTX-paralysed muscles only few fibres weakly stained with the anti-2A mAb. On the other hand, the expression of type 2A isoform was high, with the patchy distribution observed in denervated muscles, in TTX-vinblastine-blocked preparations. As reported for soleus muscle [12, 13] this suggests that the axoplasmic flow carries a factor that inhibits the expression of this isoform. Thus, the suggestion that the nerve exerts an inhibitory effect on type 2A MHC expression is not limited to slow muscle. The most significant difference we observed between slow and fast muscle regenerated in the presence of the TTX block was that the type 2A isoform was completely lacking in soleus muscle, whereas in EDL muscle it was still expressed in some fibres. It may be that in EDL muscle there are satellite cells specifically committed to differentiate into type 2A fibres, which resist the inhibitory action of axoplasmic flow. Alternatively, not all motor fibres to the muscle exert an inhibitory action.

Abbreviations

EDL: extensor digitorum longus
MHC: myosin heavy chain
TTX: tetrodotoxin
mAb: monoclonal antibody

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References

- [1] Benoit PW, Belt WD: Destruction and regeneration of skeletal muscle after treatment with a local anaesthetic, bupivacaine (Marcaine). *J Anat* 1970; 107: 547-556.
- [2] Betz WJ, Caldwell JH, Ribchester RR: Sprouting of active terminals in partially inactive muscles of the rat. *J Physiol (London)* 1980; 303: 281-297.

Fast muscle regeneration

- [3] Bigard XA, Janmot C, Merino D, Lienhard F, Guzenneec YC, d'Albis A: Endurance training affects myosin heavy chain phenotype in regenerating fast-twitch muscle. *J Appl Physiol* 1996; 81: 2658-2665.
- [4] Bigard AX, Janmot C, Sanchez H, Serrurier B, Pollet S, d'Albis A: Changes in myosin heavy chain profile of mature regenerated muscle with endurance training in rat. *Acta Physiol Scand* 1999; 165: 185-192.
- [5] Cerny LC, Bandman E: Expression of myosin heavy chain isoforms in regenerating myotubes of innervated and denervated chicken pectoral muscle. *Develop Biol* 1987; 119: 350-362.
- [6] d'Albis A, Couteaux R, Janmot C, Roulet A, Mira JC: Regeneration after cardiotoxin injury of innervated and denervated slow and fast muscles of mammals. Myosin isoform analysis. *Eur J Biochem* 1988; 174: 103-110.
- [7] Esser K, Gunning P, Hardeman E: Nerve-dependent and -independent patterns of mRNA expression in regenerating skeletal muscle. *Dev Biol* 1993; 159: 173-183.
- [8] Gorza L, Sartore S, Thornell L, Schiaffino S: Myosin types and fiber types in cardiac muscle: III. Nodal conduction tissue. *J Cell Biol* 1986; 102: 1758-1766.
- [9] Gunning P, Hardeman E: Multiple mechanisms regulate muscle fiber diversity. *FASEB J* 1991; 5: 3064-3070.
- [10] Hoh JF: Myogenic regulation of mammalian skeletal muscle fibres. *News Physiol Sci* 1991; 6: 1-6.
- [11] Kashiba H, Senba E., Kawai Y, Ueda Y, Tohyama M: Axonal blockade induces the expression of vasoactive intestinal polypeptide and galanin in rat dorsal root ganglion neurons. *Brain Res* 1992; 577: 19-28.
- [12] Megighian A, Germinario E, Rossini K, Midrio M, Danieli-Betto D: Nerve control of type 2A myosin heavy chain isoform expression in regenerating slow skeletal muscle. *Muscle & Nerve* 2001; 24: 47-53.
- [13] Midrio M, Danieli-Betto D, Esposito A, Megighian A, Carraro U, Catani C, Rossini K: Lack of type 1 and type 2A myosin heavy chain isoforms in rat slow muscle regenerating during chronic nerve block. *Muscle & Nerve* 1998; 21: 226-232.
- [14] Ribchester RR: Co-existence and elimination of convergent motor nerve terminals in reinnervated and paralyzed adult rat skeletal muscle. *J Physiol (London)* 1993; 466: 421-441.
- [15] Schiaffino S, Gorza L, Sartore S, Saggin L, Ausoni S, Vianello M, Gundersen K, Lomo T: Three myosin heavy chain isoforms in type 2 skeletal fibres. *J Muscle Res Cell Motil* 1989; 10: 197-205.
- [16] Schiaffino S, Reggiani C: Molecular diversity of myofibrillar proteins: gene regulation and functional significance. *Physiol Rev* 1996; 76: 371-423.
- [17] Sesodia S, Choksy RM, Nemeth PM: Nerve-dependent recovery of metabolic pathways in regenerating soleus muscles. *J Muscle Res Cell Motil* 1994; 15: 573-581.
- [18] Talmadge RJ, and Roy RR: Electrophoretic separation of rat skeletal muscle myosin heavy-chain isoforms. *J Appl Physiol* 1993; 75: 2337-2340.
- [19] Whalen RG, Harris JB, Butler-Browne GS, Sesodia S: Expression of myosin isoforms during notexin-induced regeneration of rat soleus muscle. *Dev Biol* 1990; 141: 24-40.
- [20] White DM, Mansfield K, Kelleher K: Increased neurite out-growth of cultured rat dorsal root ganglion cells following transection or inhibition of axonal transport of the sciatic nerve. *Neurosci Lett* 1996; 208: 93-96.
- [21] Zhuo H, Lewin AC, Phillips ET, Sinclair CM, Helke CJ: Inhibition of axoplasmic transport in the rat vagus nerve alters the numbers of neuropeptide and tyrosine hydroxylase messenger RNA-containing and immunoreactive visceral afferent neurons of the nodose ganglion. *Neuroscience* 1995; 66: 175-187.