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# Neural impacts on the regeneration of skeletal muscles★✪

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**The regeneration of skeletal muscles is a suitable model to study the development and differentiation of contractile tissues. Neural effects are one of the key factors in the regulation of this process. In the present work, effects of different reinnervation protocols (suture or grafting) were studied upon the regenerative capacity of rat soleus muscles treated with the venom of the Australian tiger snake, notexin, which is known to induce complete necrosis and subsequent regeneration of muscles. Morphological and motor endplate analysis indicated that the regenerative capacity of denervated, and thereafter surgically reinnervated muscles remains impaired compared to that of normally innervated muscles, showing differences in the muscle size, fiber type pattern and motor endplate structure, even 35 days after the notexin injection. A lack or deficiency of secreted neural factors, deterioration of satellite cells and/or incomplete recovery of the sutured or grafted nerves may be the cause of these discrepancies in the regeneration process.**

The functional diversity of skeletal muscle fibers is deeply rooted in their innervation pattern (Pette & Staron, 1990; Pette & Vrbová, 1992). Loss of innervation not only by experimental denervation, but also by accidents leads to general morphological and physiological deterioration of the affected fibers (Sunderland & Ray, 1950; Gutmann & Zelena, 1962; Borisov *et al.,* 2001; Germinario *et al.,* 2002). The superficial localization, mass and mechanical activities expose skeletal muscles and their motoneurons to

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**Abbreviations:** TNF-$\alpha$, tumor necrosis factor-$\alpha$; SERCA, sarcoplasmic/endoplasmic reticulum $Ca^{2+}$ ATPase; HE, haematoxylin-eosin; AChE, acetylcholinesterase.

different types of injuries. Injury of the brachial plexus may occur in newborns (during delivery) as well as in young adults in (motorcycle) accidents (Mackinnon, 1993; Alnot, 1995). Microsurgical protocols allow early nerve reconstruction aiming at regaining active muscle function soon after the injury. The injured part of the nerve is either replaced by a peripheral nerve (grafting), or less frequently by simple suture, or in some cases the so-called free muscle transplantation is recommended as an alternative (Berger & Brenner, 1995; Doi *et al.,* 1998). Even after a good nerve reconstruction, the muscle function remains impaired in high-level mixed-type nerve injuries. The reason for this impairment can be in part the relatively late neurotisation (3–6 months after the injury), by which time the denervated muscle fibers are severely atrophied. Moreover, the regeneration of the nerve and the reinnervation of the muscles normally need weeks or months, and afterwards adaptic axon losses may disable the perfect reinnervation (Alnot, 1995).

Injured skeletal muscle fibers undergo almost complete regeneration provided their satellite cell content was unharmed. The experimentally induced muscle regeneration processes are suitable for characterization of the events and regulation during muscle development and differentiation (Lefaucheur & Sebille, 1995; Saito & Nonaka, 1994). The Australian tiger snake venom notexin is one of the most frequently used inducers of muscle necrosis for subsequent regeneration studies (Harris *et al.,* 1975; Harris & Johnson, 1978; Preston *et al.,* 1990; Grubb *et al.,* 1991; Davis *et al.,* 1991; Vater *et al.,* 1992).

The regeneration process following notexin administration has been thoroughly characterized in the last decade, in normal and dystrophic skeletal muscles (Sewry *et al.,* 1992; Dux *et al.,* 1993; Wilson *et al.,* 1994a; 1994b; Zádor *et al.,* 1996; 1998; 1999; 2001; Mendler *et al.,* 1998a; 1998b; 2000). Molecular events of the regeneration process, such as expression of myogenic regulatory factors and some growth factors (myostatin, TNF-$\alpha$), formation of new motor endplates with reinnervation, expression and re-establishment of myosin and SERCA isoform distribution were analysed. Introduction of antisense RNAs or expression vectors into the regenerating muscles allowed the modulation of the regeneration model (Zádor *et al.,* 2002; Zádor & Wuytack, 2003).

The regenerated muscle achieved its normal morphology by the 28th day post notexin injection, although some nuclei were still in a central position in the fibers (Harris *et al.,* 1975; Harris & Johnson, 1978; Whalen *et al.,* 1990; Sesodia & Cullen, 1991). In regenerated soleus muscles the typical slow myosin isoform and sarcoplasmic reticulum structure were recovered (Whalen *et al.,* 1990; Sesodia & Cullen, 1991), although myosin and SERCA isoform composition become more uniform (Davis *et al.,* 1991; Mendler *et al.,* 1998b; Zádor *et al.,* 1998). The complete recovery of the metabolic capacity in regenerated muscles ensured the background for functional activity (Sesodia *et al.,* 1994). Although electrophysiological studies indicated that regenerated muscles were able to produce normal action potentials and contractions as soon as the newly formed motor endplates obtained their mature form (Grubb *et al.,* 1991; Whalen *et al.,* 1990), in the functional sense the recovery did not seem to be complete. Louboutin *et al.* (1995) reported that the amplitudes of contractions in the regenerated muscle remained strongly dependent on the external $Ca^{2+}$ concentration, a feature typical of neonatal muscles, instead of normal adult muscle fibers.

In the present work we explored the notexin-induced muscle regeneration of denervated/reinnervated rat soleus. In this first phase of experiments morphological changes were characterized at the light and electron microscopic levels. Moreover, the dynamics of motor endplate formation was followed during the regeneration of reinner-

vated muscles, compared to those regenerating with normal innervation. We also compared the effects of different nerve reconstruction techniques (suture or grafting) at the morphological level.

## MATERIALS AND METHODS

***Treatment of animals***. Adult male Wistar rats (250–280 g) were anesthetized with intraperitoneal injection of Nembutal. The left sciatic nerve was exposed at the proximal part of the thigh by splitting the gluteal muscle.

***Reinnervation protocols***. In the first group of animals, an approx. 12 mm nerve segment was resected and used as an autologous nerve graft. The coaptation sites were sutured by 10/0 nylon epineural sutures. The second group of rats was reinnervated by making simple suture at the proximal cutting level.

***Control animals***. In the third group of animals, a nerve segment of more than 30 mm was removed without doing nerve reconstruction.

Based on preliminary experiments, at 3 months after microsurgery the soleus muscles of the denervated, sutured or grafted animals from both the uni- and contralateral sides were removed and further processed for morphological analysis. Each group of animals contained at least 3–4 animals.

***Induction of regeneration***. In the fourth group of rats, that had been reinnervated by grafting, muscle necrosis was induced to the soleus muscle by intramuscular injection of 20 $\mu$g of notexin in physiological NaCl solution. This amount of notexin was chosen since it is known to induce complete necrosis to the muscle (Mendler *et al.*, 1998a) (see Fig. 3A). At 1, 3, 5, 7, 10, 21, 28 and 35 days after injection, animals were sacrified by an overdose of Nembutal injection, and soleus muscles of both the injected and the contralateral hindlimbs were removed, weighed and further processed for morphological analysis. At each stage of regeneration 3–4 animals were examined.

***Preparation and staining of tissue sections.*** Soleus muscles of all groups of animals (denervated, reinnervated by graft or by suture, reinnervated and regenerated muscles) were processed for light microscopical analysis. Cryostat sections of 15 $\mu$m thickness were either stained with haematoxylin-eosin (HE) or the motor endplate formation was checked by using the method of Tago (Tago *et al.*, 1986) staining the acetylcholinesterase (AChE) activity of the endplates. Some samples of grafted-regenerated soleus muscles were also processed for standard electron-microscopy (EM).

## RESULTS AND DISCUSSION

In denervated rat soleus muscles we detected pronounced and uniform atrophy (Figs. 1 and 2D) and only diffuse, if any,

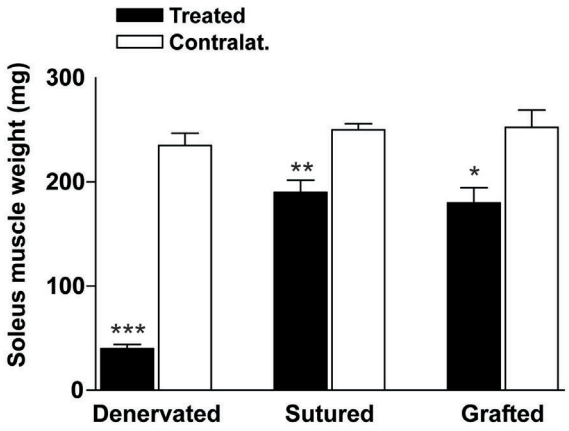


**Figure 1. Muscle weights of denervated and reinnervated (sutured or grafted) rat soleus muscles 3 months after microsurgery.**

Colums represent mean values ±S.E.M. of data obtained from 3–4 animals in each group, asterisks show significant differences compared to untreated contralateral muscles (Paired *t*-test, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$**).** Reinnervated muscles differed significantly from their contralateral ones, and also from the denervated muscles ($P < 0.001$, ANOVA). Muscle weights of the sutured and grafted muscles did not differ significantly from each other ($P > 0.05$, ANOVA).

AChE activity showing no motor endplate formation (Fig. 2H). The weights of reinnervated soleus muscles were significantly higher than of the denervated ones ($P < 0.001$, ANOVA), but they did not reach the values of the contralateral untreated muscles even after 3 months of reinnervation (Fig. 1). In all of the investigated reinnervated muscles we found atrophied fibers either in groups, characterized by more pronounced connective tissue as well (Fig. 2B insert), or interspersed (Fig. 2C) among fibers which had close to normal diameter and morphology (Fig. 2A, B, C). In line with these findings, the morphology of motor endplates was variable, they were smaller and of unmatured character in the regions of the atrophied fibers (Fig. 2F insert, G). Others also described (Ijkema-Paassen *et al.*, 2001b, Wang *et al.,* 2002) that rat soleus muscles did not regain their normal size and endplate morphology even after 21 weeks of reinnervation. Moreover, the normally slow type muscle was transformed into a predominantly fast phenotype (Ijkema-Paassen *et al.*, 2001a; 2001b; Wang *et al.,* 2002). The fast type tibialis anterior muscles showed better recovery in all the aspects investigated, suggesting that the fiber type composition, and consequently, the initial innervation pattern of a given muscle can ultimately influence the efficiency of the reinnervation later on. However, there were no data in the literature whether different microsurgical techniques, i.e. suture *versus* graft have different effects on muscle recovery. We found that the weights of sutured and grafted muscles did not differ significantly from each other ($P > 0.05$, ANOVA; Fig. 1) and the morphology was similar in both cases, at least at the light microscopical level (Fig. 2B, C, F, G). Therefore, we used grafted muscles for the regeneration studies since this technique proved to be clinically more relevant.

The regeneration process of grafted muscles showed differences compared to that of normal muscles, although – similar to the normally innervated ones – notexin induced

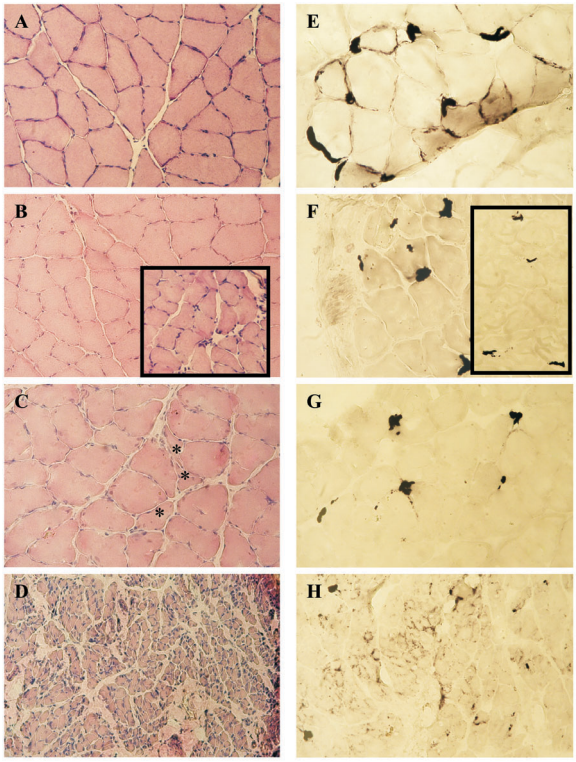


**Figure 2. Fiber and motor endplate morphology of normal, reinnervated and denervated rat soleus muscles 3 months after microsurgery.**

After removal of the muscles, cryostat sections of 15 $\mu$m thickness were either stained with haematoxylin-eosin (HE) or the motor endplate formation was checked by staining for acetylcholinesterase (AChE) activity of the endplates. **A–D**: HE staining of normal, grafted, sutured and denervated muscles, respectively. **E–H**: AChE staining of normal, grafted, sutured and denervated muscles, respectively. In grafted muscles we detected atrophied fibers and more connective tissue (B insert) besides fibers of close to normal morphology (B). This difference was also evident in the variability of the size of motor endplates (F and F insert). The morphology of sutured muscles was similar to that of grafted muscles, here we show regions with atrophied fibers interspersed among normal ones (C asterisks) with variable endplate morphology (G). Denervated muscles showed general atrophy (D) and only diffuse, if any, AChE-activity (H). Magnification 200×.

complete necrosis by day 1 (Fig. 3A). By this time muscle weights did not decrease (Fig. 4) probably because of the pronounced oedema. Three days after notexin injection the muscles were significantly smaller than the un-

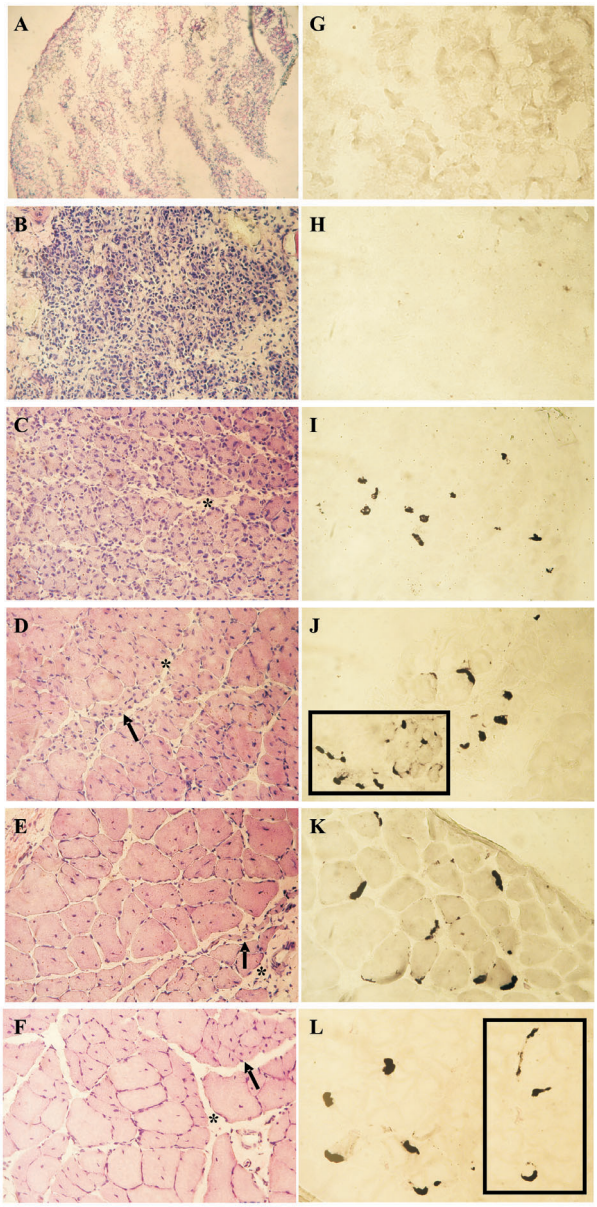


**Figure 3. Fiber and motor endplate morphology of reinnervated (grafted) rat soleus muscles regenerating from notexin-induced necrosis.**

After removal of the muscles, cryostat sections of 15 $\mu$m thickness were either stained with haematoxylin-eosin (HE) or the motor endplate formation was checked by staining for acetylcholinesterase (AChE) activity of the endplates. **A–F**: HE staining of muscles 1, 3, 5, 10, 28, and 35 days after notexin injection, respectively. **G–L**: AChE staining of muscles 1, 3, 5, 10, 28, and 35 days after notexin injection, respectively. Notexin induced complete necrosis by day 1 (A) destroying virtually all fibers. Three days after notexin injection mononucleated cells filled up the injured muscle (B). Most of the mononucleated cells had already fused to form new myotubes by day 5 (C). From this stage on, however, connective tissue seemed to be more abundant throughout the regeneration process (C–F, asteriks). Abnormal and pronounced variability of the fibre size is the characteristic feature of the 7- and 10 day-regenerated muscles (D, arrow shows smaller fibers). Even after 28 and 35 days of regeneration, the fibre size variability was still present (E, F arrows show smaller fibers) and more than 80% of the fibers still contained centrally located nuclei. Notexin treatment destroyed all the motor endplates by day 1 (G) and we did not see any signal of motor enplate formation until day 3 (H). The first new motor endplates reappeared by day 5 after necrosis (I), at a similar time to those of normally innervated regenerated muscles. However, their morphology seemed to be more variable even at late stages of regeneration (J, K, L) showing smaller and in some cases fragmented motor endplates (J, L inserts). Magnification 200×, except Fig. 3A (magnification 40×).

treated ones (Fig. 4), and mononucleated cells filled up the whole cross-section area of the injured muscle (Fig. 3B). Like in the normally innervated muscles, most of the mononucleated cells had already fused to form new myotubes by day 5 (Fig. 3C). From this stage on, however, connective tissue seemed to be more abundant in the reinnervated muscles throughout the regeneration process (Fig. 3C–F). Abnormal and pronounced variability of the fibre size was the characteristic feature of the 7 and 10 day-regenerated/reinnervated muscles (Fig. 3D). Even after 28 and 35 days of regeneration (Fig. 3E, F), the fibre size variability was still present and more than 80% of the fibers still contained centrally located nuclei, much more than found in normally innervated regenerated muscles (Mendler *et al.*, 1998a). These findings, together with the significantly lower muscle weights at this late stage of regeneration (Fig. 4), suggest that a significant number of fibers were not able to perfectly regenerate/differentiate.

Although notexin treatment destroyed all the motor endplates by day 1 (Fig. 3G, H), the first new motor endplates reappeared at a similar time (by day 5 after necrosis, Fig. 3I) as those of normally innervated regenerated muscles. However, their morphology seemed

to be more variable even at late stages of regeneration showing smaller and in some cases fragmented motor endplates coupled to smaller/less regenerated fibers (Fig. 3J, K, L with inserts).

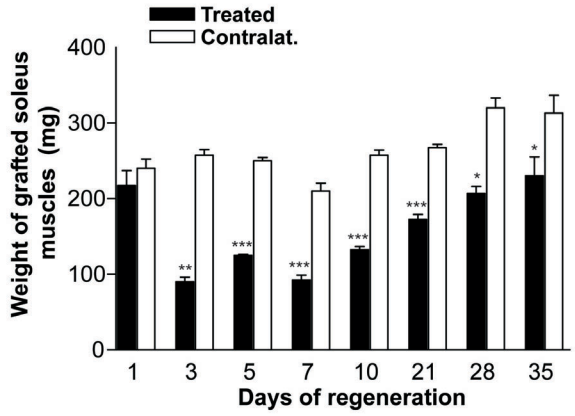


**Figure 4. Muscle weights of reinnervated (grafted) rat soleus muscles regenerating from notexin-induced necrosis.**

Columns represent mean values ±S.E.M. of data obtained from 3–4 animals at each stage of regeneration (1–28: days after notexin administration), asterisks show significant differences compared to untreated contralateral muscles (Paired *t*-test, $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$). By day 3 after notexin treatment, the muscles became significantly smaller than their untreated counterparts. Thereafter, muscle weights increased until the end of the examined period of regeneration, but even at that time they were smaller than the contralateral ones.

At the ultrastructural level, grafted regenerated muscles showed serious abnormalities of mitochondria by day 35 after notexin treatment (not shown).

In this work, our aim was to characterize the muscle regeneration capacity of reinnervated muscles. Up to now, no data were available in the literature in this regard. It is well known that denervated muscles have impaired regenerative capacity, which is most pronounced during the second phase of regeneration, i.e. the newly formed primitive fibers are not able to differentiate to reach their normal size (Sesodia & Cullen, 1991). Still, it is debated whether satellite cells, the main sources of muscle regeneration (Mauro, 1961; Bishoff, 1993; Asakura *et al.*, 2002), are inactivated by denervation (Maier *et al.*, 2002), or on the contrary, they become more active upon denervation (Nnodim, 2001). This author found in the specific androgen dependent levator ani muscle of male rats that the activation of satellite cells caused by denervation could be prevented by castration, indicating that the effects of denervation can be modulated by other (humoral) factors as well.

Our morphological results are in line with the original hypothesis that the regenerative capacities of reinnervated muscles might be impaired. One reason could be that since reinnervation is not complete, satellite cells of more atrophied regions might be of lower activity (number?) than those of the normal ones. However, the question seems to be even more complicated. As cited earlier, other groups (Ijkema-Paassen *et al.*, 2001b) described that reinnervated muscles show high proportion of endplates of abnormal morphology, which we could also confirm in our experiments. Yet we do not know whether this pattern of "abnormality" will be recapitulated in the course of regeneration when the newly formed endplates are established after complete necrosis. In theory, it cannot be ruled out that the necrotized muscle sends cues for the reinnervating axons which might modify the reinnervation pattern of the regenerating reinnervated muscle. If so, it could well be that the fiber-type composition also shows changes after regeneration of reinnervated muscles. Investigation of the myosin and SERCA isoforms at both mRNA and protein levels and their expression pattern in tissue sections, compared to normally innervated regenerated muscles (Zádor *et al.*, 1998; 1999; Mendler *et al.*, 1998b), would at least partly answer this question. Moreover, the regulatory molecules like myogenic regulatory factors or myostatin (Mendler *et al.*, 1998a; 2000; Zádor *et al.*, 1999) involved in the differ-

entiation of the fibers, or any element of the calcium-calcineurin signaling cascade may also show changes, which we would also like to explore in further experiments.

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