The histochemical profile of the rat extensor digitorum longus muscle differentiates after birth and dedifferentiates in senescence

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Age dependent motor unit dedifferentiation is a key component of impaired muscle function in advanced age. Here, we tested the hypothesis that rat muscle histochemical profile during the lifespan of an individual has an age-specific pattern since comprehensive longitudinal studies of muscle differentiation after birth and dedifferentiation in advanced age are scarce. Our results show that extensor digitorum longus muscle (EDL) is comprised only of two fiber types after birth, type slow-oxidative (SO) and type SDH-intermediate (SDH-INT), the latter being indicative for the presence of polyneuronal innervation. In contrast to the constantly growing cross-sectional area of the muscle fibers, a dramatic decrease in SDH-INT proportion occurs between day 14 and 21 after birth resulting in a complete loss of fiber type SDH-INT at the age of 90 days ($p<0.05$). At the age of 270 days, the fiber type composition of rat EDL dedifferentiates as shown by the reappearance of the SDH-INT type with a further increase at the age of 540 days ($p<0.05$). These changes in histochemical fiber type spectra are brought about by fiber type conversion within the fast twich fibers. The findings of the present study provide further evidence that fiber type conversion is a basic mechanism leading to motor unit differentiation and dedifferentiation during ontogenesis. Fiber type conversion shows a distinct time specific pattern and is also characteristic for motor unit regeneration after peripheral nerve repair. Factors that influence fiber type conversion and thereby motor unit organization may provide a future therapeutic option to enhance the regenerative capacity of motor units.

Key words: ageing physiology, motor unit, muscle histochemistry.

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Impaired muscle function is a key feature in various diseases involving the motor unit or one of its components (α-motoneuron, axon and its innervated muscle fibers). Peripheral nerve injury and/or the physiologic ageing process represent two insults to the integrity of the motor unit. In addition, a profound change at the functional level within the motor system is a characteristic feature during growth. Subsequently, de- and redifferentiation processes of motor units take place and they represent key determinants for motor unit reorganization and thus the final muscle function.

During the first postnatal weeks, muscle fibers innervated by more than one α-motoneuron (polyneuronal innervation) undergo a process termed *synapse elimination* that decreases the size of motor units, and induces the adult distribution of motor unit sizes (Lomo 2003). Age-dependent decline of motor performance is a well described phenomenon and is partially caused by degeneration of motoneurons and muscle fibers (Hashizume 1988; Luff 1998). Single denervated muscle fibers are recaptured by regenerating motor axons. This results in an age-dependent remodelling process of motor units in man and animals (Campbell 1973; Kanda 1989). In addition, age is an independent risk factor for adverse clinical outcome after reconstruction of a peripheral nerve (Allan 2000). Muscle palsy after lesion of a peripheral nerve repair is accompanied by a profound shift in muscle fiber type composition paralleled by reorganization of spinal cord motoneurons, representing motor unit deorganization (Wasserschaff 1990; Lehnert 2003). Age-dependent changes as well as regeneration after peripheral nerve repair represent de- and reinnervation processes. Within these three conditions (newborn, advanced age, peripheral nerve repair) motor unit organization primarily affects both its morphological and functional properties (Kadhiresan 1996; Rafuse 1998).

The morphological properties of motor unit

organization are mirrored in the histochemical profile of a given muscle, reflecting its functional abilities and metabolic properties. Therefore, analysis of the histochemical profile represents an important tool to study muscle regeneration (Karpati 1967; Pette 1985; Lehnert, 2003). Previously, we studied the reorganization of the histochemical profile of the rat extensor digitorum longus (EDL) muscle after either end-to-end repair or grafting of the peroneal nerve up to 15 months post-repair (Lehnert, 2003; Lehnert 2004). This reorganization was characterized by the appearance of a fiber type that was not present in those rats that did not undergo nerve repair. The fiber type was named SDH-INT. The percentage of the SDH-INT fibers mirrors the amount of motor unit destabilisation within the EDL muscle and represents a suitable tool to quantify effects of various interventions on motor unit de- and reorganization. Motor unit reorganization after nerve repair was also affected by increasing age (Lehnert, 2004). These results show that age with its influence on the regenerative capacity of motor unit organization is an important factor that determines the progress and the outcome of diseases affecting the motor system. A detailed analysis of rat histochemical profile during the ageing process provides a more thorough understanding of nerve-muscle interdependence and its influence on physiological and pathophysiological (i.e. nerve repair) conditions. However, due to variabilities in fiber type nomenclature and various fiber type classification systems, histochemical data from comprehensive longitudinal studies of rat EDL histochemical profile starting in the newborn until advanced age has not been published. Therefore, we tested the hypothesis that muscle histochemical profile during the lifespan of an individual has a time specific pattern from maturation until advanced age.

Materials and Methods

Surgical procedure

A total of 29 EDL muscles from male Sprague Dawley rats were studied at ages of 0, 5, 14, 21, 30, 60, 270 and 540 days. The rats were housed under physiologic light cycles and fed ad libitum with standard laboratory food and tap water. Under pentobarbital anaesthesia (6.4 mg/100 g body weight i.p.), extensor digitorum longus muscles were excised. All muscles were immediately frozen in n-hexane, cooled in dry ice at -70°C and stored at the same temperature until further processing. We wanted to avoid unpredictable effects on the musculature dependent on disease and disuse that frequently occur in animals at an advanced age, so we decided not to study animals older than 540 days. Surgical procedures were approved by the animal rights committee of the University of Saarland at Homburg/Saar, Germany.

Histochemistry

To obtain a representative picture of the histochemical profile of the muscle studied, the central portion of the muscle was chosen for examination (Larsson 1986); 10 μm transverse serial sections were cut in a cryostat and air dried. One of the sections was assessed for myofibrillar ATPase activity following acidic preincubation at pH 4.3 according to a procedure previously described by Brooke and Kaiser (Brooke 1970). Positively stained fibers were classified as slow-twich fibers as opposed to fast-twich fibers, which showed no mATPase staining at pH 4.3 (Figure 1, panel B, (Brooke 1970)). In the subsequent serial tissue-section, the relative oxidative potential of both the slow twich and the fast-twitch fibers was determined by the activity of succinate dehydrogenase (SDH, Figure 1, panel A, (Peter 1972; Nemeth 1981)). The slow twich fibers showed a high activity for SDH and were classified based upon their high oxidative potential as SO (slow-oxidative). The fast-twitch fibers (no mATPase activity at pH 4.3) were further subdivided into fast oxidative glycolytic (FOG) fibers and fast glycolytic (FG) fibers based upon their intense (FOG) or strongly reduced (FG) staining intensity for SDH (Figure 1, panel A, C (Horak 1983; Armstrong 1984)). A fast twich fiber type (no mATPase activity at pH 4.3) with intermediate SDH staining intensity (SDH-INT) as previously described (Nacimiento 1993; Constantinidis 2001; Lehnert, 2003; Lehnert, 2004) was also identified (Figure 1, panel D). As in previous studies, all sections were typed by one experienced examiner. Accordingly, the visual allocation of the muscle fibers to the different fiber types based on the myofibrillar ATPase activity and SDH activity was highly reproducible. We decided not to include additional methods of fiber typing (i.e. based on the expression of myosin heavy chain (MHC) isoform expression) to keep

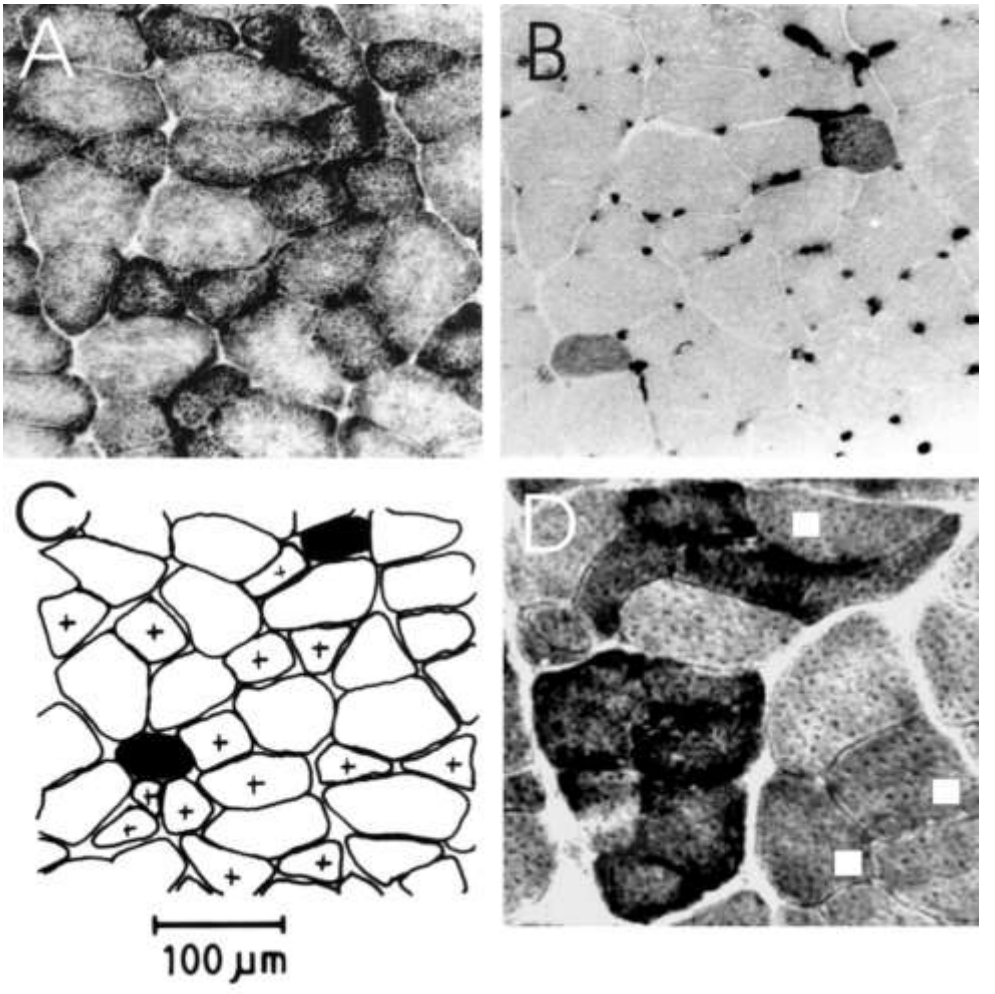

Figure 1. Illustration of the histochemical classification system. After mATPase stain at pH 4.3, the two dark fibers are classified as fiber type I, light fibers are classified as fiber type II (panel A). In the subsequent SDH stain (panel B) fibers with low and high staining intensity for SDH are shown. The drawing in panel C indicates fiber type SO (black fibers, positive for mATPase stain at pH 4.3 and high SDH activity), fiber type FOG (+marked fibers, negative for mATPase stain at pH 4.3 and high SDH activity) and FG (fibers not marked, negative for mATPase stain at pH 4.3 and low SDH activity). SDH-INT fibers shown intermediate staining intensity for SDH (marked with white rectangle, panel D).

the results comparable to previous studies (Nacimiento, 1993; Constantinidis, 2001; Lehnert, 2003; Lehnert, 2004).

Morphometry

Four muscle fiber types were identified after histochemical staining as described. An overall muscle cross-section was drawn from each muscle using a side tube projection from the microscope (Olympus BH2, Figure 1, panel C). The numbers of each muscle fiber type were then counted and their cross-sectional areas were measured using a digitizing tablet. Since a compartimentalized innervation in rat EDL is present under physiological conditions, we chose to evaluate whole muscle cross-section (Balice-Gordon 1988). This non-random distribution of fibers belonging to the same histochemical type is also present during the physiologic ageing process (Ansved 1991). Accordingly, all fibers of the different fiber types are recorded and sampling errors due to fragmentary evaluation of the muscle cross-section were avoided (Coggeshall 1992).

Statistical analysis

The percentage of each fiber type per animal was transformed by arcus sinus transformation to obtain a normal distribution. Homogenity of group variances were verified and differences between the various age groups were evaluated by one-way analysis of variance with Tukey post-hoc analysis. All values are given as a mean with standard error of the mean (S.E.M.).

Results

Total muscle fibre count was not statistically different according to age (Table 1).

Age dependent changes in fibre type composition of EDL

Until 14 days after birth, EDL is almost completely composed of fiber type SDH-INT with a subsequent sharp and significant decrease compared to muscles from 21 days old rats ($p<0.05$; Figure 3). This decline continues until the disappearance of fiber type SDH-INT at the age of 90 days. The SDH-INT fibre-type reappears to a small extent at 270 days (n.s.) and even increases at 540 days ($p<0.05$; Figure 3). The fall in the percentage of SDH-INT (68.8%) fibers is paralleled with a rise of the proportion of fiber type FOG (32.7%) and FG (33.6%), which both significantly increase from 14 to 21 days after birth ($p<0.05$; Figure 3). The increase of the proportion of fiber type FOG continues until 30 days, whereas the first peak in the proportion of fiber type FG is reached at 60 days ($p<0.05$; Figure 3). The reappearance of the fiber type SDH-INT at 270 days is paralleled by a significant decline of the fiber type FOG ($p<0.05$; Figures 2, 3) whereas the fiber type FG remains stable. However, with the further increase of the SDH-INT portion at 540 days, the fiber type FG also decreases. The proportion of the fiber type SO shows a small but significant increase at 14 compared to 5 days ($p<0.05$, Figure 3). Its contribution to the complete fiber type spectra does never exceed 13% throughout the whole observation period. These data show, that the fiber type composition of the EDL is age-dependent. A sharply differentiated fiber type triade of EDL at 90 days is present only during a limited time period during ontogenesis.

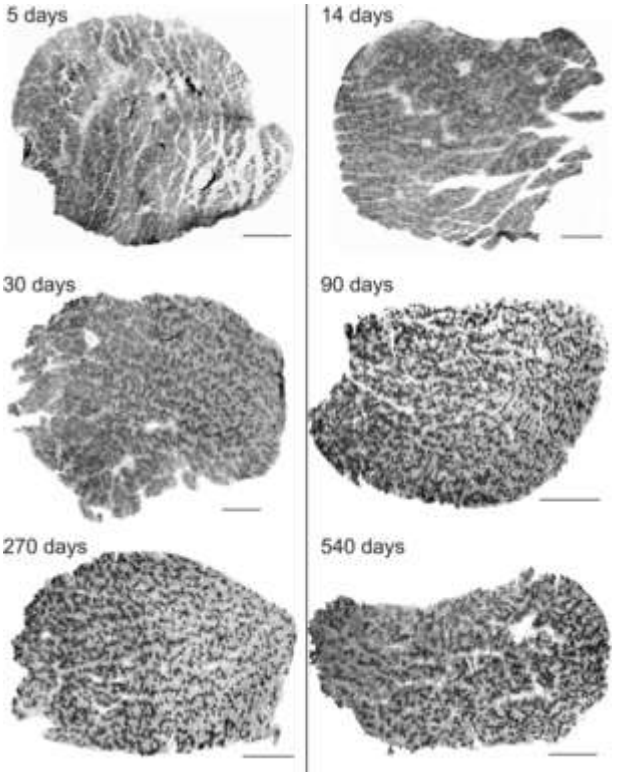

Figure 2. Profound alterations of the EDL histochemical profile during ontogenesis. Representative succinate dehydrogenase-stained whole muscle cross-sections (as described in Materials and Methods) of extensor digitorum longus muscle are shown. Note the emergence of a distinct histochemical pattern starting between 14 and 30 days after birth. Also, accumulation of fibers from the oxidative muscle fiber groups in the medio-dorsal portion of the muscle is obvious. Bar is 200 µm for 5, 14, 30 days and 1 mm for 90, 270 and 540 days.

Table 1. Morphometric data of all groups studied. The fibre type composition (%) and the cross-sectional area (CSA [µm²]) are given for each muscle fiber type according to age. Values from 90 days control and values from 540 days as published previously (Lehnert, Steudel, Marzi, Mautes 2003).

Age [days]	*Number of fibres*	*Fibre type SO*		*Fibre type FOG*		*Fibre type FG*		*Fibre type SDH-INT*	
%	*CSA [µm²]*	*%*	*CSA [µm²]*	*%*	*CSA [µm²]*	*%*	*CSA [µm²]*	*%*	*CSA [µm²]*
1	2880±215	0.8±0.2	1.6±0.2	--	--	--	--	99.2±0.2	0.8±0.1
5	2977±448	0.8±0.3 #	1.3±0.3	--	--	--	--	99.2±0.3	1.0±0.1
14	3254±445	4.1±3.1	3.1±0.5	0.9±0.5 #	2.6±0.4	--	--	95.0±3.7 #	2.5±0.3
21	4117±542	4.3±0.6	3.6±0.3	37.1±4.6 #	3.4±0.3	31.0±4.9	4.9±0.7	27.6±7.7	4.8±0.5
30	3788±287	5.0±0.3	6.3±0.4 #	51.7±1.8	6.5±0.4 #	28.1±1.7 #	12.4±1.0 #	15.0±0.7 #	10.4±0.7 #
60	3681±191	3.0±0.2	12.1±0.3	39.5±3.6	15.4±1.2	50.1±1.8	31.2±2.1	7.4±2.2 #	23.1±1.8 #
90	3916±432	1.9±0.3	10.7±0.4 #	45.7±0.6 #	12.8±0.5 #	52.5±0.9 #	25.9±1.3 #	-- #	-- #
270	2788±177	2.8±1.3	18.5±1.5	34.6±1.6	25.1±1.2	55.7±2.0 #	56.3±2.9	6.8±0.8 #	37.5±2.0
540	3206±138	2.3±1.1	19.2±0.6	26.4±1.5	27.8±1.0	47.9±4.1	57.9±2.3	19.7±2.9	42.0±2.1

#: p < 0.05 vs. subsequent age group. Data are given as mean ± standard error of the mean, n = 4-6 per group.

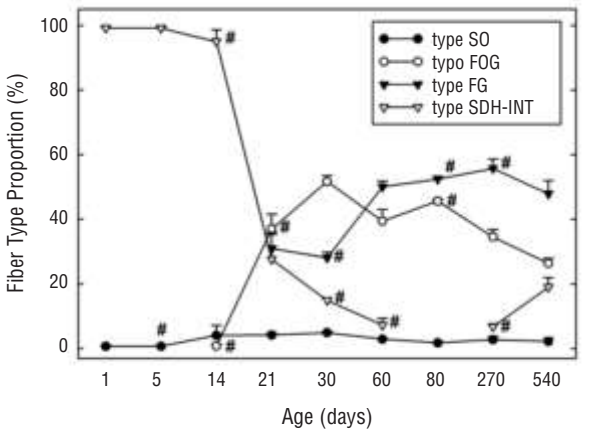

Figure 3. Morphometry of time-dependent changes in fiber type composition. Serial sections of whole muscle cross sections were stained for succinate dehydrogenase and m-ATPase (pH 4.3). Based on the enzymatic activities four muscle fiber types were identified and counted (as described in Materials and Methods); #: $p<0.05$ vs. subsequent age group. N is 4-6 per group.

Age dependent changes in the cross-sectional area of EDL

The cross-sectional area (CSA) all four fiber types show a remarkable similar pattern. After a slow and continuous progression of the CSA within the first time after its appearance, a significant increase of CSA in all four fiber types occurs at the age of 60 compared to 30 days ($p<0.05$, Figure 4). The CSA remains mainly unaffected between 60 and 90 days. At 270 days, again a substantial increase independent from the fiber type can be observed ($p<0.05$, Figure 4). These data show that all fiber types in rat EDL are subject to uniform, age-dependent variations with respect to CSA. In contrast, proportions of individual fiber types show a specific, fiber type dependent pattern (Figure 3).

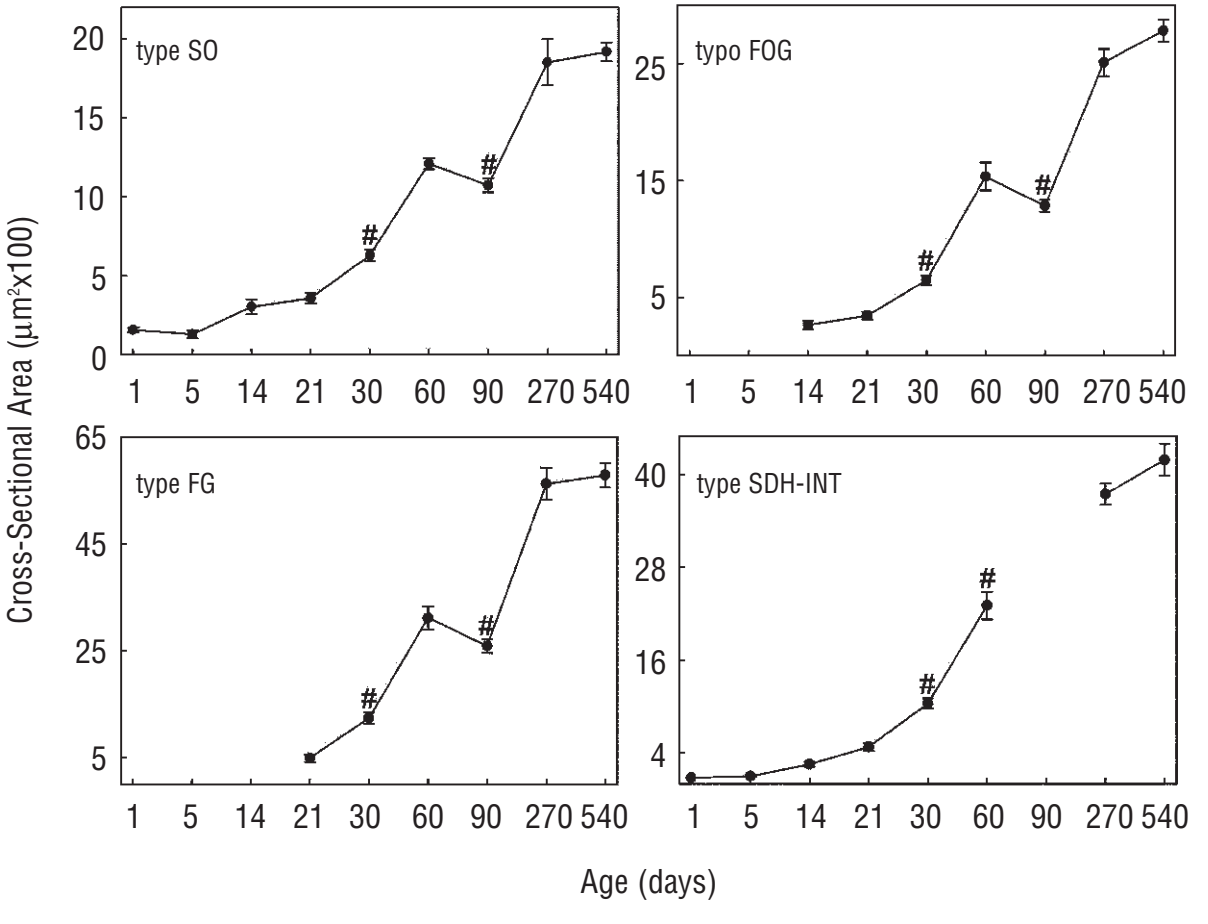

Figure 4. Morphometry of changes in cross-sectional area according to age. The cross-sectional area of all individual fibers was determined (as described in Materials and Methods); #: $p<0.05$ vs. subsequent age group; N=4-6 per group

Discussion

The present study provides further insight into the differentiation process of rat EDL from postnatal development to its dedifferentiation with advancing age. It demonstrates the presence of a distinct maturation period from birth up to the age of 90 days. Until the age of 90 days, the muscle histochemical profile is comprised of four fiber types (fiber type SO, FOG, FG and SDH-INT). Afterwards, fiber type SDH-INT is completely lost, a process that results in a characteristic fiber type triade and the level of the highest differentiation is reached. This represents an only transient and unstable state. Already at the age of 270 days a dedifferentiation process evolves, characterized by the reappearance of the the SDH-INT fiber type. The proportion of fiber the type SDH-INT further increases with advancing age (18 months).

The total muscle fiber count helps, together with the cross-sectional area (CSA) to explain growing or wasting of total muscle mass under various conditions. There is ongoing debate in the literature on the number of muscle fibers with respect to postnatal development and also with advancing age. Some studies report an increase in total fiber number after birth (Yoshioka *et al.,* 1982), while other studies, including the present one, don't find differences (Rowe *et al.,* 1969). In senescence, total fiber number and cross-sectional area of EDL and its fiber types was stable in rats up to 18 months, confirming results reported in our study (Alnaqeeb 1987; Brown 1987).

Fiber type differentiation based on SDH activity

In addition to SDH staining, other investigators also evaluated activities of the glycolytic metabolism of muscle fibers (Maltin 1989; Punkt 2004). The results clearly demonstrate, that differentiation of metabolic fiber types is feasible both using markers of oxidative and glycolytic metabolism. In a study in rat tibialis anterior fiber types, distribution and proportion of fiber types classified by differences in their oxidative enzyme histochemistry was identical to that observed when fibers were classified using glycolytic enzyme histochemistry (Pullen 1975). Numerous additional possibilities for classification of fiber types exist, as recently reviewed (Meola 2005). However, based on the activity of mATPase and on the activity of SDH on serial tissue sections, description of fiber types SO, FOG and FG is also established in the literature (Soukup 1979; Horak 1983; Alnaqeeb, 1987; Fazarinc 1995).

Factors contributing to the shift in histochemical profile at day 21

The strong predominance of the fiber type SDH-INT up to the third postnatal week (Figures 2, 3) in our study confirms earlier results (Punkt 1993). This study describes with regard to SDH-reactivity an undifferentiated fast-twich fiber type accounting for 94.2% of all muscle fibers at the age of 14 days. Similarly, a differentiation of fiber types into FOG and FG fibers at day 21 was reported, the time that coincides with a sharp decline of SDH-INT fibers in our study (Alnaqeeb, 1987). Before birth, no fiber types can be discerned by staining for neonatal, slow and fast MHC isoforms. Starting the first day after birth, fast and slow MHC can be differentiated in EDL (Punkt, 1993; Punkt, 2004). The neonatal isoform of MHC is eliminated up to day 21 and an adult electrophoretic pattern of MHC isoforms is present (LaFramboise 1990). The reorganization of muscle histochemical profile is mirrored by profound changes of the fiber type composition at this crucial developmental stage. One important feature of motor units in newborn is the presence of polyneuronal innervation as shown by an *in vivo* staining procedure and also verified by multiple end plate potential recordings in newborn animals (Redfern 1970; Balice-Gordon 1993). Loss of this polyneuronal innervation is an active process that takes approximately 3 weeks, starting after birth (Tweedle 1981). Other mechanisms that may contribute to the postnatal fiber type differentiation include the increase in presynaptically dependent release properties such as quantal content, spontaneous release frequency and evoked potential amplitude. These properties change particularly in the rat EDL 3 weeks after birth (Bewick 2004). With regard to physiological muscle function around 3 weeks after birth, rats leave the nest, start to maintain posture and movements of hindlimbs become necessary. These needs require subspecialized fiber types that evolve around that time in our and also in other studies (Alnaqeeb, 1987; Punkt, 1993) (Figures 2, 3). Taken together, the decrease in the proportion of fiber type SDH-INT (Figures 2, 3) during growth is paralleled by the loss of polyneuronal innervation, by changes in the patterns of presynaptic activity and by changes in the animals behaviour and exigencies on the motor system of the

growing individual. These factors may contribute to the profound shift in histochemical profile in the early individual development seen in our study.

Histochemical development of motor unit organization with advancing age

The reappearance of the SDH-INT type at the age of 270 days indicates early reorganization of the motor unit, a finding that was confirmed by Kugelberg (1976) with a reorganization of soleus muscle fibers at that age (Kugelberg 1976). In our series age-induced alterations of muscle fiber type composition further increases at the age of 540 days (Figure 3).

The changes in the proportions of fiber type SDH-INT are exclusively brought about by fiber type conversion within the fast twich fibers. The SO fiber type is stable throughout the complete study period. The fiber type SDH-INT shows similar histochemical (SDH activity) and morphological (cross-sectional area) properties as the fiber type IIX (Larsson 1991b). Changes in energy metabolism, such as increase or decrease of oxidative capacity (reflected in the SDH measurements) precede the following changes in myosin heavy chains composition (Pette, 1985). MHC isoforms of rat tibialis anterior muscle show an age-related switch to the MHC fiber type IIX where they constitute the predominant fiber type. In addition, the contraction and the half-relaxation times of the isometric swich increased (Larsson 1991a). Also in rat EDL, an age related fast-to-slow shift of within the pattern of the fast MHC isoforms occurs (Skorjanc 1998). This also confirms the results of the present study that quantifies increasing destabilization of fiber type spectrum starting as early as 270 days and further increasing at 540 days. The changes observed within the fast twich fiber group are paralleled by age-induced alterations of various components of the motor unit such as loss of α-motoneurons, axonal demyelinization and Wallerian degeneration and expression of neural cell adhesion molecules, changes that are also described after repair of injured nerves (van Steenis 1971; Hashizume, 1988; Larkin 2003). Interstingly, the same fiber types are affected both after peripheral nerve repair as in the physiologic ageing process and changes in histochemical profile are brought about by fiber type conversion within the fast twich fibers (Lehnert, 2003). These results show, that changes in the histochemical profile both after peripheral nerve repair and during the physiologic ageing process have relevant similarities. So it is tempting to speculate that comparable mechanisms might be involved in motor unit reorganization both after peripheral nerve repair and during the physiologic ageing process with potent therapeutic implications.

Taken together, the results of the current study shed further light on the age dependent change of motor unit organization in rat EDL. The dynamic process of these changes is outlined, providing a platform to better understand the pathophysiology of muscle fiber de- and reinnervation processes. Identifying mechanisms that lead to distinct fiber type spectra in the postnatal period might provide a potential therapeutic approach to promote motor unit reorganization that takes place after peripheral nerve repair.

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