Uncoordinated expression of the main contractile and relaxation proteins in pathological skeletal muscles

Ph.D. thesis

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Articless related to the subject of the Thesis

I. **Szabó A**, Wuytack F, Zádor E. (2008) The Effect of Passive Movement on Denervated Soleus Highlights a Differential Nerve Control on SERCA and MyHC Isoforms, *J Histochem Cytochem* **56(11):** 1013-1022.
IF: 2.823

II. Rácz G, **Szabó A**, Vér A, Zádor E. (2009) The slow sarco/endoplasmic reticulum Ca^{2+} -ATPase declines independently of slow myosin in soleus muscle of diabetic rats. *Acta Biochim Pol.* **56(3):** 487-493.
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Article not related to the subject of the Thesis

I. Tóth N, **Szabó A**, Kacsala P, Héger J, Zádor E. (2008) 20-Hydroxyecdysone increases fiber size in a muscle-specific fashion in rat. *Phytomedicine.* **15(9):** 691-698
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Abbreviations

AGPC	**a**cid **g**uanidinium thiocyanate-**p**henol-**c**hloroform method
BCA	**bic**inchoninic **a**cid assay (method)
BSA	**b**ovine **s**erum **a**lbumin
cDNA	**c**omplementary **d**eoxyribo**n**ucleic **a**cid
CSA	**c**ross **s**ectional **a**rea
DAB	**di**amino**b**enzidine
DHPR	**d**y**h**ydro**p**yridine **r**eceptor
DM	diabetes mellitus
dNTP	**d**eoxy**n**ucleoside **t**ri**p**hosphate mix (dATP:dCTP:dGTP:dTTP=1:1:1:1)
ECL	**e**lectro**c**hemi**l**uminescence
GAPDH	**g**lycer**a**ldehyde-3-**p**hosphate **d**e**h**ydrogenase
HD	**h**indlimb **d**enervation
HRP	**h**orse**r**adish **p**eroxidase
IRS	**i**nsulin **r**eceptor **s**ubstrate
MEF-2	**m**uscle **e**nhancing **f**actor**-2**
mRNA	**m**essenger **r**ibo**n**ucleic **a**cid
MyHC	**my**osin **h**eavy **c**hain
myoD	**myo**genic **d**ifferentiation (factor)
NFAT	**n**uclear **f**actor of **a**ctivated **T**-cells
PBS	**p**hosphate **b**uffered **s**aline
PI3-K	**p**hosphatidyl**i**nositol **3-k**inase
PVDF	**p**oly**v**inylidene **di**f**l**uoride membrane
RNS	**r**eactive **n**itrogene **s**pecies
ROS	**r**eactive **o**xigene **s**pecies
RR	**r**yanodine **r**eceptor
RT PCR	**r**everse **t**ranscriptase **p**olymerase **c**hain **r**eaction
SD	**s**elective **d**enervation (of soleus)
SE	**s**tandard **e**rror of mean
SERCA	**s**arcoplasmic/**e**ndoplasmic **r**eticulum **C**a^{2+} **a**denosine triphosphatase
SR	**s**arcoplasmic **r**eticulum
stz-rats	**st**rep**z**otocin-induced diabetic rats.
TT	**t**ransversal **t**ubule

Summary

The sarcoplasmic reticulum Ca2+ ATPase (SERCA) isoforms are normally expressed in coordination with the corresponding myosin heavy chain (MyHC) isoforms in the fibers of skeletal muscle but this coordination is often disrupted in pathological conditions. Here we used three models, the selective denervated soleus (SD), the hindlimb-denervated soleus (HD) and the soleus of streptozotocin-induced diabetic rat (stz-rat) to study such dyscoordinated expressions.

SD allowed more passive movement of the soleus, less muscle atrophy, faster transition from slow-to-fast MyHC isoforms and less coordinated expressions of the MyHCs and SERCAs than HD did. Among the investigated isoforms, generally, the expression of the slow-twitch type SERCA2a was the less dependent while the slow-twitch type MyHC1 was the most dependent on innervation. Our study also showed that passive movement is able to ameliorate denervation-induced atrophy, but also accentuates dyscoordinated expression of the corresponding slow and fast MyHC and SERCA isoforms.

In stz-rats, the soleus muscle showed peripheral neuropathy and decreased SERCA2a level in type I (slow-oxidative) fibers, till the expression of the corresponding slow MyHC1 did not change compared to the controls. No difference in the mRNA and protein levels of SERCA and MyHC isoforms was found, except that the SERCA2a protein specifically declined in stz-rats compared to the controls. This was enough to disrupt the coordinated expression of SERCA2a and MyHC1 in the diabetic soleus. The results are in line with the observations of other laboratories that regulators of the Ca-homeostasis may adapt faster to type I diabetes than the contractile elements.

In conclusion, the slow-to-fast transformations are different in each of the three pathological models. This is in line with previous findings that the expression of MyHC and SERCA isoforms has a multiple control and dyscoordinated in various conditions.

Introduction

Skeletal muscle is characterized by the type of its fibers. The quality of muscle fibers is determined by functional properties, summerized as the "phenotype". As a simplified view of the phenotypic heterogeneity, four major fiber types are distinquished in mammals (recent review by Schiaffino and Regiani, 2011). These are characterised by the dominantly expressed myosin heavy chain proteins (one of the main contractile elements), the twich properties and the metabolic functions (Table 1) (Schiaffino *et al.,* 1989; Schiaffino and Regiani, 1996; Baldwin and Haddad, 2001). The type I fibers contain MyHC1, are slow-twich, fatigue resistant and oxidative in respect of metabolic properties (characterized by high succinate dehydrogenase (SDH) activity). The type II fibers are fast-twitch and divided into three subtypes: IIA, IIX/D and IIB. Type IIA fibers express MyHC2a, are fast-twitch with oxidative metabolism. The dominant contractile protein in type IIB fibers is MyHC2b, these have the fastest shortening speed and the most glycolitic metabolism. Type IIX or IID fibers contain MyHC2x/d and are intermediate because they have shortening velocity between type IIA and type IIB fibers, and their metabolism is mainly glycolitic

Table 1. Skeletal muscle fiber types and their dominant contractile proteins, shortening speed and metabolism and their corresponding relaxation proteins

Fiber type	**I**	**IIA**	**IIX/D**	**IIB**
Expressed myosin heavy chain	MyHC 1	MyHC 2a	MyHC 2x/d	MyHC 2b
Shortening speed	slow	fast	faster	fastest
Type of metabolism	oxidative	oxidative	glycolitic	glycolitic
Expressed SERCA isoform	SERCA2a	SERCA1a	SERCA1a	SERCA1a

The MyHC isoforms are the most frequently used phenotypical markers of skeletal muscle (Schiaffino *et al.,* 1989; Baldwin and Haddad, 2001), but not the only molecules that determine the muscle phenotype (Schiaffino and Reggiani, 1996; Hämäläinen and Pette, 1997; 2001). For example, while the MyHC-s control the speed of contraction, the rate of relaxation depends on the SERCA isoforms (Fig. 1.; Schiaffino and Reggiani, 1996; 2011; Berchtold *et al.,* 2000).

In mammals there are three SERCA genes expressed, each has alternative mRNAs and protein isoforms (recent review by Brini and Carafoli, 2009). SERCA1 is a skeletal muscle specific gene which has one adult and one neonatal mRNA with open reading frames different at the 3' ends (Brandl *et al.,* 1986, 1987; Korczak *et al.,* 1988). The adult SERCA1a is expressed mainly in fast, type II fibers while the neonatal SERCA1b protein is present explicitely in the neonatal or regenerating myotubes (Zádor *et al.,* 2007) which are the developmental forms of the adult muscle fibers. SERCA2 has four mRNAs, one of them the SERCA2a is expressed in slow skeletal muscle (in type I fibers) and in the heart ventricle, the other mRNAs uniformly translate SERCA2b, an ubiquitously expressed Ca^{2+} pump. Lately, a non muscle specific SERCA2c transcript has been discovered in human. The SERCA3 gene is not expressed in skeletal muscle therefore its mRNAs and proteins are not detailed here.

The corresponding isoforms of slow and fast MyHC and SERCA are usually coexpressed in the muscle fibers suggesting that they are subject to a common regulatory mechanism (Kandarian *et al.,* 1994; Talmadge *et al.,* 1996; Hämäläinen and Pette, 1997). This regulatory mechanism has two main components; one is determined by the position of the muscle, the other one is the electric component of innervation (Kalhovde *et al.,* 2005). The muscle position influences the potential of satellite cells that are the tissue stem cells in skeletal muscle and contribute to the maintanence/regeneration of fibers by providing myonuclei (Zammit *et al.,* 2006). Unlike in regeneration, in normal muscle the nerve activity is obviously more frequent than the activation of satellite cells. Therefore the type of innervation is a major determinant of muscle type in normal use. However, it is a further question, if there is a difference in nerve effect in normal and regenerating muscle.

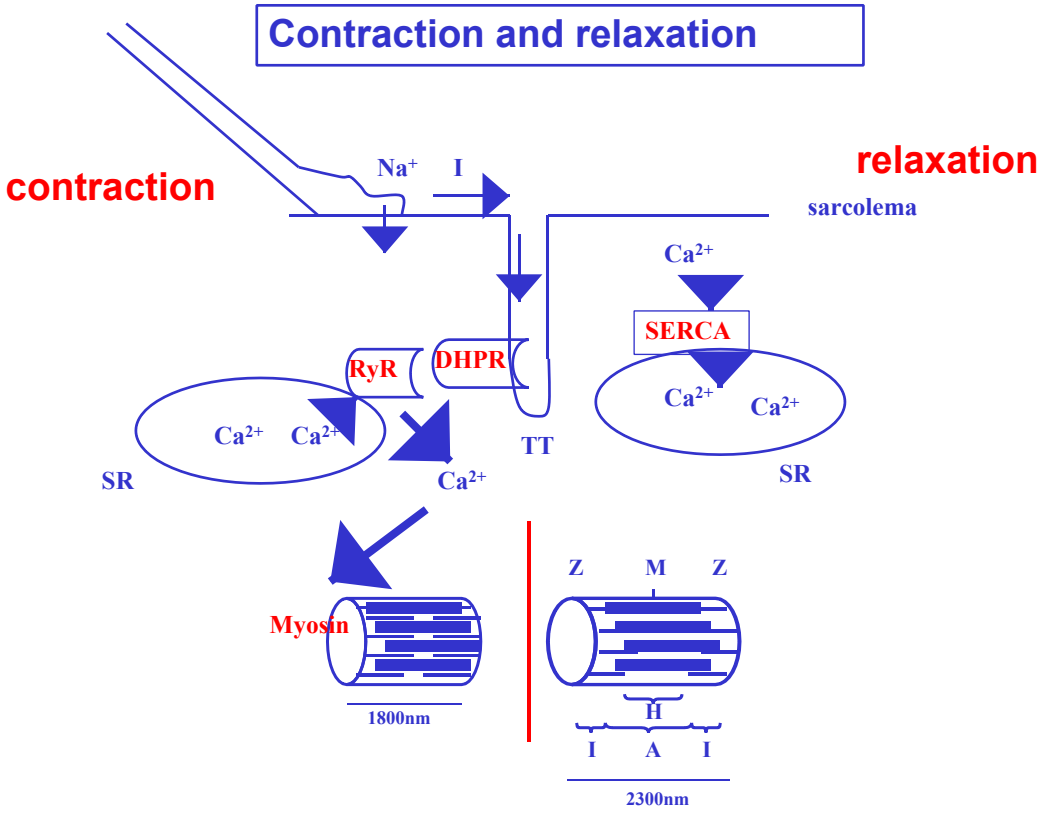


Figure 1: *The scheme of contraction and relaxation of skeletal muscle.* In the left part of the figure, the contraction is initiated by the membrane potential change generated by Na^+ influx through the acetylcholine receptor. This electric current induces conformation change of the dihydropyridine receptor (DHPR) in the transversal tubule (TT) which opens the ryanodine receptor (RyR) in the terminal part of the sarcoplasmic reticulum (SR). The RyR eliberates Ca^{2+} to the sarcoplasm which renders the actin (thin line as microfilaments) and myosin (thick lines as macrofilaments) in the sarcomeres to contract (bottom of the figure). In case of relaxation (right half of the figure), the sarcoplasmic Ca^{2+} is pumped back to the SR by SERCA and this makes possible the temporal disconnection of myosin head from actin, therefore relaxation. Z line, M, H, I and A zones in relaxation and the sarcomer length in both stages are indicated.

Denervation models

In the denervated regenerating soleus, the slow SERCA2a isoform persists whereas the corresponding slow MyHC1 level declines, showing that the expression of SERCA2a is not directly dependent on innervation (Zádor and Wuytack, 2003). In accordance with this, in regenerating soleus the inactivation of Ras (Zádor and Wuytack, 2003) or calcineurin (Zádor *et al.,* 2005), the two pathways mediating the nerve effect, induced a decline in the expression of MyHC1, but left the expression of SERCA2a unchanged. Although the nervous control of muscle gene expression may differ in the regenerating versus normal muscle (Moreno *et al.,* 2003), a number of studies showed that the expression of the corresponding SERCA and MyHC isoforms are also dissociated in paralyzed cat and rat soleus (Zhong *et al.,* 2002; Talmadge and Paalani, 2007), in overloaded soleus (Awede *et al.,* 1999) and in denervated soleus muscles (Leberer *et al.,* 1986; Hämäläinen and Pette 2001; Roy *et al.,* 2002).

We hypothesized that in the normal soleus muscle the impact of nerve activity on the expression of SERCAs differs from that on the corresponding MyHC isoforms. In order to test this hypothesis, we denervated the hindlimb of the rat (HD). To further refine our study, we thereupon compared this intervention with selectively denervating the soleus (SD) thereby leaving the other hindlimb muscles intact (Zádor and Wuytack, 2003). We assumed that in SD soleus the adaptation to passive movement might affect the levels of the MyHC and SERCA isoforms differently if they are each under a different control. We assessed the effects of our interventions after no longer than two weeks. A longer denervation affects the normal regulatory pathways, *e.g.* after 15 days of denervation the expression of the slow myosin is not dependent on calcineurin in the regenerating soleus (Launay *et al.,* 2006) like it does after one week denervation (Serrano *et al.,* 2001). We expected a slightly different dissociation in the expression of MyHC and SERCA isoforms in HD and SD soleus that might be also different from that in the normal muscle. In any case, the comparison of these two types of denervation has not been made in the literature; therefore our study might be interesting for both basic research and muscle therapy.

Diabetic model

We used a different pathological status of the soleus affected by type I diabetes mellitus as another model to check our hypothesis suggesting that the regulation of SERCA and MyHC expressions is different.

There are two main types of diabetes mellitus (DM). Type I DM occurs when pancreas β-cells are destroyed, therefore total lack of insulin affects the fatty tissue and the skeletal muscle which have insulin-dependent glucose uptake. In case of type II DM the constantly high blood glucose level induces increased insulin excretion of the pancreas β-cells. Due the hyperinsulinaemia, the fatty tissue and the skeletal muscle develop insulin resistance, decreasing the efficiency of insulin in uptake of glucose from the blood. The decreased insulin efficiency makes the β-cells to excrete even more insulin. At the end of this circle the β-cells become exhausted, the insulin level falls and becomes unable to maintain normal blood glucose level.

The streptozotocin-treatment of rodents is a modell of DM I (reviewed in Szkudelski, 2001). Stretozotocin (STZ) is a fungal toxin which damages β-cells. Skeletal muscle is a major target of insulin. Decline of insulin level observed in type I diabetes restricts glucose uptake and severely affects metabolism, function and morphology of muscle (Klueber and Feczko 1994; Aughsteen *et al.,* 2006, reviewed by Sun *et al.,* 2008). Stz-rats develop peripheral neuropathy (Klueber and Feczko, 1994; Snow *et al.,* 2005) and a slow-to-fast transition of fiber types in the soleus (Punkt *et al.,* 1999; Snow *et al.,* 2005). The conversion from slow to fast muscle phenotype declines the level of slow myosin heavy chain (MyHC1) and increases the levels of fast myosins (MyHC2a, MyHC2x and MyHC2b) (Pette and Staron, 2000). The coordinated expression of the corresponding SERCA isoforms (Table 1) is functionally relevant and characteristic to this conversion. However, current research has shown that factors controlling the levels of myosins and SERCAs are different in both normal and pathological conditions (Zador and Wuytack, 2003; Zador *et al.,* 2005; Talmadge and Paalani, 2007). By the same token, regulators of the Ca^{2+} homeostasis (like SERCAs) respond relatively rapidly to blood glucose level in skeletal muscle. The calcineurin-NFAT pathway is down regulated, and the DNA binding of NFAT (nuclear factor of activated T-cells) and MEF-2 (muscle enhancing factor-2) is lower in rat skeletal muscle three weeks after stz-treatment (Costelli *et al.,* 2007). Moreover, insulin receptor substrate 1 and 2 (IRS-1 and IRS-2) increases binding to SERCA1

and SERCA2 in normoglycemic rat after insulin stimulation and phosphorylation by insulin receptor kinase (Algenstaedt *et al.,* 1997). This suggests that SERCAs are targets of insulin action and hints at a direct link between the insulin-dependent and Ca^{2+}-signaling pathways. The MyHC1 level decreases in the rat soleus at 2–3 months after STZ treatment (Punkt *et al.,* 1999; Snow *et al.,* 2005), but no parallel detection of MyHC and SERCA isoforms has been presented in the same diabetic muscle, so we looked at them also in this pathological model.

Aims

Since both MyHC and SERCA levels are regulated by signal pathways which probably change in adaptation, we wanted to describe the patterns of slow-to-fast transformation in the soleus by determining mRNA, protein levels and fiber-expression of MyHC1, MyHC2a, SERCA2a and SERCA1 (1) in selectively denervated and hindlimb denervated soleus after 3, 7 and 14 days and (2) in diabetic rat soleus four weeks after stz-treatment. Putting together the results we aimed to extend the present view about the regulation of MyHC and SERCA isoforms in skeletal muscle.

Materials and methods

Animals and treatments

Experiments with animals of the denervation models were approved by the Ethics Committee of Animal Treatment of the Medical Faculty of the University of Szeged (XV./03923/001/ 2006). Experimental protocols for stz-treatment were in accordance with the guidelines of the Committee on the Care and Use of Laboratory Animals of the Council on Animal Care at the Semmelweis University of Budapest, Hungary (TUKEB 99/94).

Selective and hindlimb denervation

Male Wistar rats 3 months of age and weighing 280-350g were used. The rats were anaesthetized with an intraperitoneal injection of 1ml of 4% chloral hydrate/100g body weight and treated the left hindlimb. Approximately 1 cm of the sciatic nerve high in thigh was cut out in the hindlimb-denervation (HD) group. In the selectively-denervated (SD) group, 0.3 cm of the soleus nerve was cut out. After denervation, the soleus muscles were dissected at 3, 7

or 14 days and frozen in isopentane cooled by boiling liquid nitrogen. Normal muscles were gained from untreated animals. n=3 in all groups except in normal group, where n=6. The muscles were kept at -70˚C until use. We also used the soleus of normal and HD 200-215g female Wistar rats to compare our results with those of Schulte *et al. (*1994).

Streptozotocin-induced diabetes

Rats with this treatment were prepared in the laboratory of Dr. Ágota Vér, (Institute of Medical Chemistry, Molecular Biology and Pathobiochemistry, Semmelweis University). In case of hyperglycaemic model the experiments were performed on 4-week-old male Wistar rats (weighing 170 ± 40 g). Rats were housed at a constant temperature (20 ˚C), humidity, 12 h light–dark cycle, and were allowed free access to standard chow and water. Rats were rendered diabetic with streptozotocin (65 mg/kg i.v., Sigma Chemical Co.) dissolved in 0.1 M citrate buffer (pH 4.5). Only stz-treated animals with plasma glucose concentrations above 15 mmol /l were considered diabetic and included in the study. Vehicle-treated rats served as controls. Four weeks after the induction of DM (defined as day 0) the animals were anaesthetized (40 mg/kg pentobarbital sodium, Abbott Laboratories) and sacrificed by decapitation. Muscles, blood and post mortem urinary samples were collected for further investigations. The soleus muscles were dissected and frozen in isopentane cooled in liquid nitrogen and stored at –80 °C. Nonfasting serum glucose concentration was measured using a kit from Roche Diagnostics, Ltd.

Ratio RT-PCR

RNA extraction and reverse transcription were carried out as described previously (Zádor *et al., 1*996). The extraction of RNA was carried out with the shorter version of AGPC method (Gauthier *et al.,* 1997). The muscle was homogenized in 0.5ml solution D /100mg muscle. (Solution D: 4M guanidinum thiocyanate, 25mM sodium citrate, 0.5% N-lauroylsarcosine, 0.1M 2-mercaptoethanol) Every 0.5ml of homogenate was dispensed into separate Eppendorf tubes, 50µl 2M sodium acetate pH4, 0.5ml water-saturated phenol and 0.2ml chlorophorm:i-amylalcohol (49:1) was added and mixed to each tube. The tubes were kept on ice for 15 minutes then centrifuged at 10 000g for 10 minutes at room temperature. The aquosus

phases were transferred to sterile centrifuge tubes. 2 volumes of 96% ethanol were added to each tube then vortexed. The tubes were centrifuged at 10 000g for 10 minutes then the supernatants were removed. The pellets were washed in 70% ethanol then dried.

Total RNA level was determined from its UV-absorption at 260nm and 280nm using a NanoDrop spectrophotometer. Primers and PCR conditions were as in Table 2 (Zádor *et al., 1*996; Mendler *et al., 1*998; Fenyvesi *et al., 2*004). The cDNA was transcribed from RNA extracts which contained 1µg total RNA. In case of DM model the same primers were used to amplify cDNA of both SERCA1 and SERCA2 in one PCR mix. The resulted amplification was divided into aliquots which were digested with either *Sty*I (hydrolysing *SERCA2*) or *Trpu*9 (hydrolysing *SERCA1*) and a control digestion with both restriction enzymes was also applied. The PCR cycles were adjusted to the logarithmic/linear amplification phase. The PCR products were analyzed on a 6% (wt/wt) polyacrylamide gel. 20µl of amplified cDNA was applied on each lane. RNA levels were deduced from the bands on the ethidium-bromide stained gels quantified by Gel Doc 2000 (Bio-Rad Laboratories, Hercules, CA) and Quantity

Table 2

Primers and cycle numbers, denaturing, annealing, and extension temperatures (each applied for 1–1–1 min) used in PCR

Primer	Sequence	Fragment size (bp)	PCR cycles	Temperatures (°C)
MyHC I	5'-acagaggaagacaggaagaacctac-3' 5'-gggcttcacaggcatccttag-3'	288	15	95–60–72
MyHC IIa	5'-tatcctcaggcttcaagatttg-3' 5'-taaatagaatcacatggggaca-3'	309	20	95–55–72
MyHC IIx	5'-cgcgaggttcacaccaaa-3' 5'-tcccaaagtcgtaagtacaaaatgg-3'	121	20	95–58–72
SERCA 2a	5'-ctccatctgcctgtccat-3' 5'-gcggttactccagtattg-3'	231	22	94-55-72
SERCA1a/ SERCA1b	5'-ttccatctgcctgtccatgtc-3' 5'-ctggttacttccttctttcgtctt-3'	248/206	21	94-60-72
SERCA1/ SERCA2	5'-gac/tgagtttggggaacagct-3' 5'-gaggtggtgatgacagcagg-3'	194	21	94-60-72

GAPDH	5'-tcctgcaccaccaactgcttagcc-3' 5'-tagcccaggatgccctttagtggg-3'	376	22	94-60-72

One software. The ratio of the SERCA transcripts was estimated from the level of the undigested fragments of the different restriction enzyme reactions. We normalized the mRNA levels to the levels of GAPDH mRNA then, because the RT was made on 1 μg of RNA, multiplied by the number of μgs of total RNA (Table 3). In this way the mRNA levels of the whole muscle were calculated.

Protein determination

Total protein levels were measured by the BCA method using the NanoDrop spectrophotometer. The crude homogenate made in the first step of the SERCA extraction was used to determine the total protein level.

Immunoblotting

SERCA and MyHC protein isoforms were measured in extracts of the same muscles. For SERCA determination immunoblots from the mitochondrial-microsomal (sarcoplasmic) fraction were used as described (Zádor *et al., 1*998). The muscles were homogenized in 2.5 ml of 20% sucrose, 5 mM HEPES (pH 7.5) and proteinase inhibitor cocktail (Roche) in the cold room. To extract myosin the pellets of the first centrifugation (1000g x 10 min) were used (Hämäläinen and Pette 1997). Amounts of extracts corresponding to equal parts of the muscles were loaded on each lane of the gel both for SERCA and myosin analysis. The loaded extracts contained different amounts of proteins i.e. less in the extract of denervated than in the extract of control muscles. Gel loading was controlled after blotting onto PVDF membrane (Immobilon-P, Millipore) by Ponceau staining. The primary antibodies used in immunoblot analysis were BA-D5 (mouse, 1:100) for MyHC1, SC-71 (Schiaffino *et al., 1*989) (mouse, 1:100) for MyHC2a, A3 (Zubrzycka-Gaarn *et al.,* 1984) (mouse, 1:50) for SERCA1 and R-15 (Wuytack *et al.,* 1989) (rabbit, 1:5000) for SERCA2a. then with HRP-conjugated 2nd antibodies and were visualized by Ni-enhanced DAB staining (Zádor *et al., 1*998) or, in case of MyHC2a, by the ECL-method and quantified by densitometry on Gel Doc 2000 (Bio-Rad). Two control samples were loaded on each gel as inner standard to make the result of different blots comparable.

Immunocytochemistry

Frozen sections were taken from the central part of the muscles and immunostained as described in (Zádor *et al.,* 1998) Serial cryosections of 20 μm thickness were cut from each block and processed for immunostaining. Sections were incubated in 1% BSA and 10% normal rabbit or goat serum in PBS for 20 min to block nonspecific binding sites. Sections were incubated overnight with primary antibodies to MyHC1 (BA-D5, mouse, 1:50, Schiaffino *et al.,* 1989), MyHC2a (SC-71, mouse, 1:20, Schiaffino *et al.,* 1989), SERCA1 (A3, mouse, 1:20, Zubrzycka-Gaarn *et al.,* 1984) and SERCA2a (R-15, rabbit, 1:400, Wuytack *et al.,* 1989) and then with the secondary antibody (peroxidase-conjugated rabbit anti-mouse immunoglobulins, Dako, 1:200 or goat anti-rabbit, Sigma, 1:200) for 30 min. The immunocomplexes were visualized by DAB staining of peroxidase activity in the presence of 0.006% H_2O_2. Control sections were immunostained in the absence of primary antibody.

The fiber cross-sectional areas (CSAs) were calculated on hematoxylin-eosin sections by the Olympus DP-soft software, v3.2 (Olympus, Hamburg, Germany).

Statistics

All statistical analyses were made by the Prism 3.0 software. We used unpaired t-tests to determine the significant differences. We considered $p<0.05$ significant, $p<0.01$ very significant and $p<0.001$ highly significant. 2–4 animals were used for the mRNA studies, 3–4 animals for the immunoblots and 3 animals for the immunohistochemistry were analysed, at least one hundred fibers were inspected for fiber types from each muscle.

Results

Denervation models

Fresh weight, fiber cross-sectional area, total RNA and total protein levels

The muscle weight, fiber cross-sectional area (CSA), the protein and RNA content were measured in HD and in SD soleus muscles. In both conditions of denervation, the fresh weight of the muscles decreased from day seven onwards (Table 3), however this decrease was less pronounced in SD than in HD muscles on day 14 ($p<0.05$). Also, the fiber cross-

sectional area was smaller compared to normal muscle at all stages in both types of denervation (Table 3). Upon SD, this decrease was larger after day 3, whereas upon HD it was more pronounced after day 7 and 14.

The RNA content decreased in HD soleus at day 7 and 14 while in SD muscles it decreased only after two weeks. Interestingly, in SD muscles, the RNA level increased first at day 3 and 7 compared to the level in HD muscle (Table 3).

The total protein levels also decreased after 14 days in both types of denervation but more dramatically upon HD than SD.

All together, the fresh weight, the fiber cross-sectional area, the total RNA and protein contents were more reduced after two weeks in HD than in SD soleus.

Table 3

Muscle mass, fiber cross-sectional areas, total RNA and total protein levels during both types of denervation. The values are means ± SEM. *, ** and *** is p<0.05, 0.01 and 0.001 compared to normal level, respectively. +, ++ and +++ is p<005, 0.01 and 0.001 compared to hindlimb denervated muscle of the same day, respectively.

Denervation type	**N**	**3dHD**	**7dHD**	**14dHD**	**3dSD**	**7dSD**	**14dSD**
Muscle mass (mg)	145.60 ± 8.46	122.50 ± 5.43	91.50 ± 2.00*	58.86 ± 3.65*	127.90 ± 3.64	114.90 ± 10.37*	89.17 ± 12.90* $^{+}$
Fibre CSA (µm²)	3,782 ± 62.16	3,546 ± 9.20**	1,975 ± 5.69***	1,475 ± 21.91***	3,179 ± 62.18*** $^{+++}$	2,233 ± 37.25*** $^{++}$	1,783 ± 32.11*** $^{+++}$
Total RNA content (µg)	113.40 ± 4.71	80.37 ± 7.61	45.74 ± 10.86**	27.46 ± 7.74***	131.30 ± 13.05^{+}	105.80 ± 9.96^{++}	45.58 ± 12.55**
Total RNA/ muscle (µg/mg)	0.699 ± 0.035	0.601 ± 0.056	0.514 ± 0.116	0.404 ± 0.087*	0.984 ± 0.112^{+}	0.825 ± 0.062	0.614 ± 0.080^{+}
Total protein content (mg)	34.73 ± 3.38	31.93 ± 4.39	31.19 ± 3.79	10.89 ± 0.48***	38.31 ± 2.96	39.78 ± 5.51	19.54 ± 0.84*$^{+++}$
Total protein/ muscle (mg/mg)	0.2705 ± 0.01687	0.285 ± 0.028	0.331 ± 0.042	0.204 ± 0.0113	0.317 ± 0.029	0.413 ± 0.035 *	0.223 ± 0.008

The mRNA levels

The slow-twitch MyHC1 mRNA level (Fig. 2A) declined in both types of denervation. This started already from day 7 in HD and somewhat later on day 14 upon SD. The level of the slow-twitch muscle-specific SERCA2a mRNA did not respond much to either form of denervation. Only on day 7 of SD (Fig. 2B) it was significantly elevated above normal. The mRNA level of the fast oxidative MyHC2a (Fig. 2C) changed differently in both types of denervation: it did not deviate significantly from the normal level in the HD group, while in the SD muscles it increased on days 3 and 7 compared to the HD muscles on the same days.

The level of the fast SERCA1 mRNA (Fig. 2D) was significantly increased at day 3 in HD soleus and at day 3 and 7 in SD. Since SERCA1 exists in two splice variants: a neonatal SERCA1b isoform (lacking a 42-bp alternative exon) and an adult SERCA1a form, we also analyzed the level of both mRNAs separately, using ratio RT PCR (Zádor *et al., 1*996). Both SERCA1a and SERCA1b mRNAs were amplified by the same pair of primers. The fragment amplified from SERCA1a was 42-bp longer than that of SERCA1b. Interestingly, whereas the level of the adult SERCA1a mRNA did not increase significantly in any of the denervated muscles, the mRNA level of the neonatal SERCA1b was higher than normal in all stages of both treatments, except the 14th day of SD muscles. The increase of SERCA1b mRNA levels was higher in SD than in HD soleus muscles during the first week.

The MyHC2x mRNA levels (Fig. 2E) showed a pronounced increase in both types of denervations, most remarkably on day 3 and 7 in SD, when it was significantly higher than in the HD muscles.

The levels of GAPDH mRNA did not change during the denervations (Fig. 2F) so it could be used as an adequate reference control level for total mRNA.

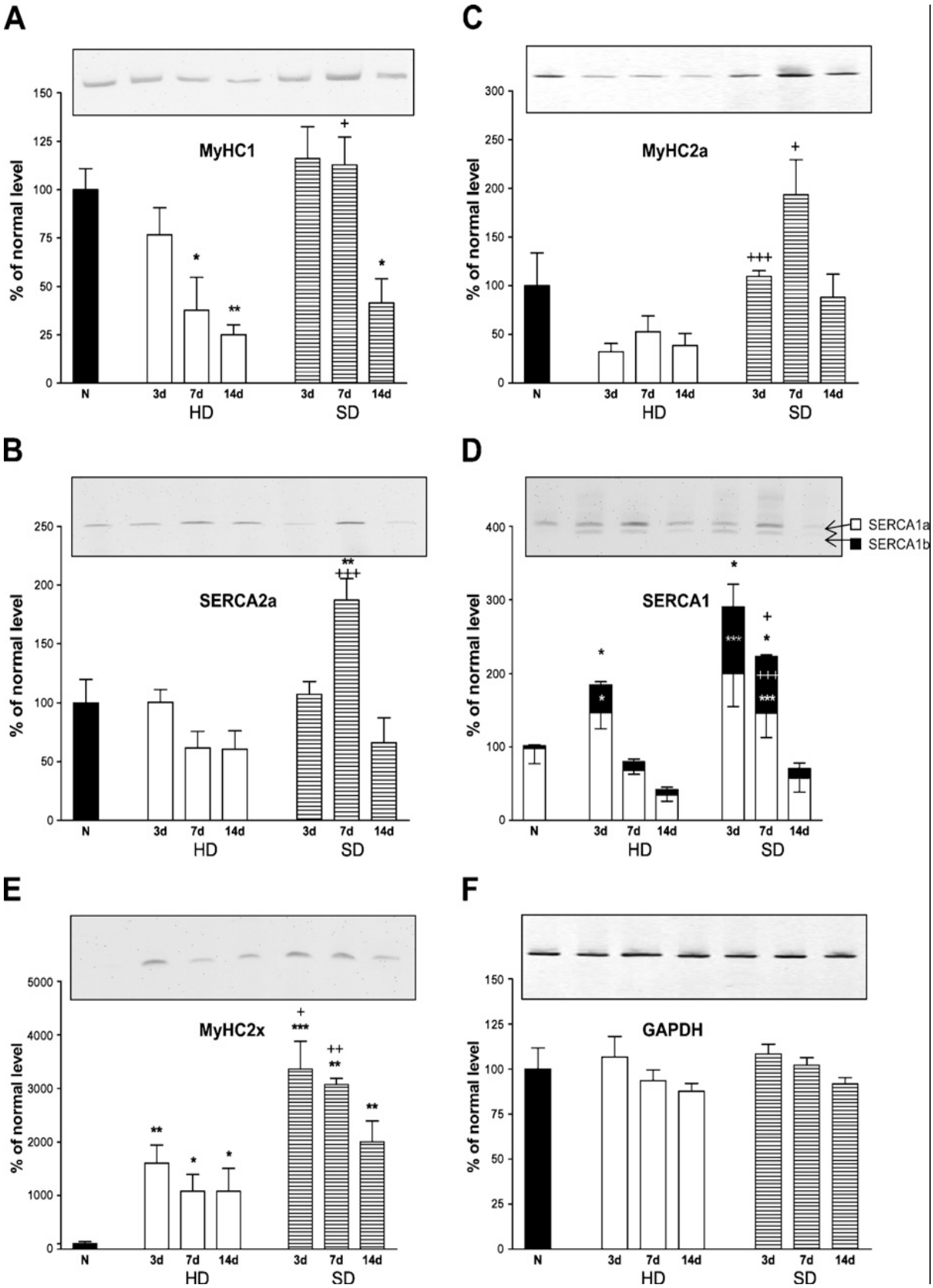

A
MyHC1
% of normal level
N
3d
7d
14d
HD
SD
C
MyHC2a
B
SERCA2a
D
SERCA1
SERCA1a
SERCA1b
E
MyHC2x
F
GAPDH

Figure 2

Figure 2: mRNA levels of MyHC and SERCA isoforms normalized to their respective GAPDH levels in hind-limb-denervated (HD) and selectively denervated (SD) soleus. (A) The decline of the MyHC1 mRNA level sets in earlier in HD than in SD. (B) The level of SERCA2a mRNA is only changed significantly on day 7 in SD. (C) The MyHC2a mRNA level does not increase in any of the denervations compared to normal level but in the first week there is a higher level in SD than in HD. (D) The ratio of the adult SERCA1a (grey part of the bar) and the neonatal SERCA1b (black part) mRNA is indicated within the bars showing the total SERCA1 mRNA level. (E) The MyHC2x mRNA level (a common marker of denervation) increases in HD and even more in SD. (F) The internal control GAPDH mRNA levels. Vertical bars give mean±SE, *, ** and *** are $p<0.05$, $p<0.01$ and $p<0.001$ compared to normal level, respectively. +, ++ and +++ are $p<0.05$, $p<0.01$ and $p<0.001$ compared to the HD of the same day. In (D) symbols above the bars refer to the total SERCA1 mRNA levels; symbols within the bars refer to the isoforms indicated by the color of the bar.

Protein levels

The MyHC1 protein level (Fig. 3A) declined significantly only at day 14 of HD, but it decreased remarkably in all stages of SD, therefore its loss was slower in HD than in SD muscles.

The SERCA2a protein levels (Fig. 3B) were not significantly altered in any of the types of denervations. Of note, similar results were obtained from 14-days HD soleus of female rats of lower weight (Fig. 4).

However at day 3, the MyHC2a protein levels (Fig. 3C) showed a slight tendency to rise in both denervations and showed a significant decrease at day 14 in HD and at day 7 and 14 in SD compared to the previous stages of the same denervation.

The total SERCA1 (SERCA1a + SERCA1b) protein levels (Fig. 3D) dropped significantly only at day 14 of HD, but it did not change in SD soleus muscles.

Figure 3: MyHC and SERCA protein levels of soleus muscles after HD and SD. (A) The MyHC1 protein level decreases more quickly in SD than in HD (B) The SERCA2a protein level is constant in both HD and SD. (C) The MyHC2a protein level does not change compared to normal level but at day 14 of HD and day 7 of SD it is less than in the previous stages of the same denervation type. (D) The SERCA1 protein level decreases only at day 14 of HD. Symbols are as in Fig 2. except ◦ and ◦◦◦ are

p<0.05 and 0.001 compared the previous stage of the same denervation type.

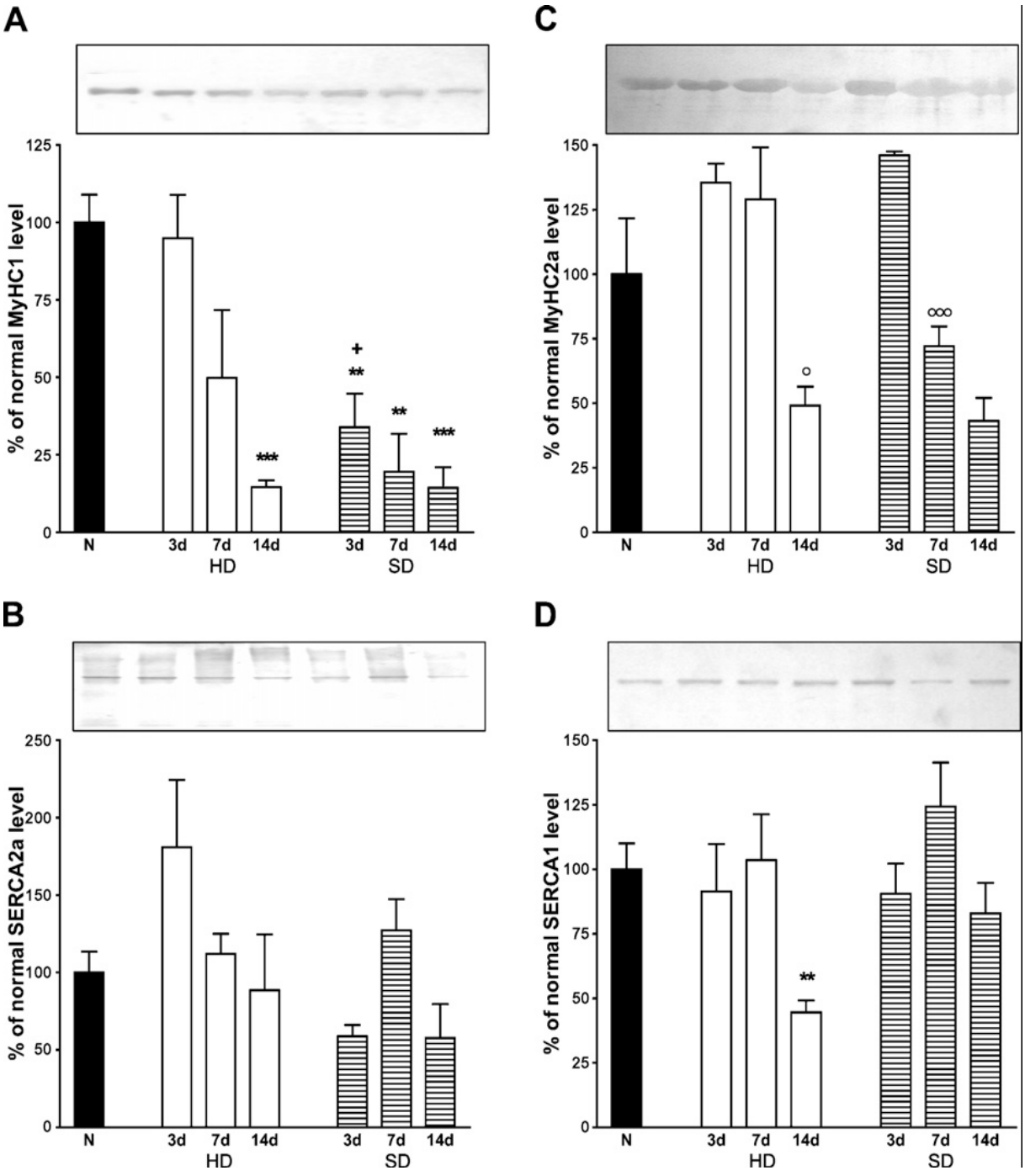


Figure 3

Viewing the literature one may suggest that our results are at variance with the results of Schulte *et al. (*1994) who found a nerve dependence of SERCA2a.

We used other methodology and rats of different sex and weight than Schulte *et al. (*1994), therefore we also analyzed female rats weighing 200g using our method, to make the comparison more adequate. In this cases the SERCA1 protein level was significantly lower (-41.81%, p=0.0361) after 14 days in HD than in normal muscles (Fig. 4). So the difference from our results was probably due to the methodology as it is detailed it in the discussion.

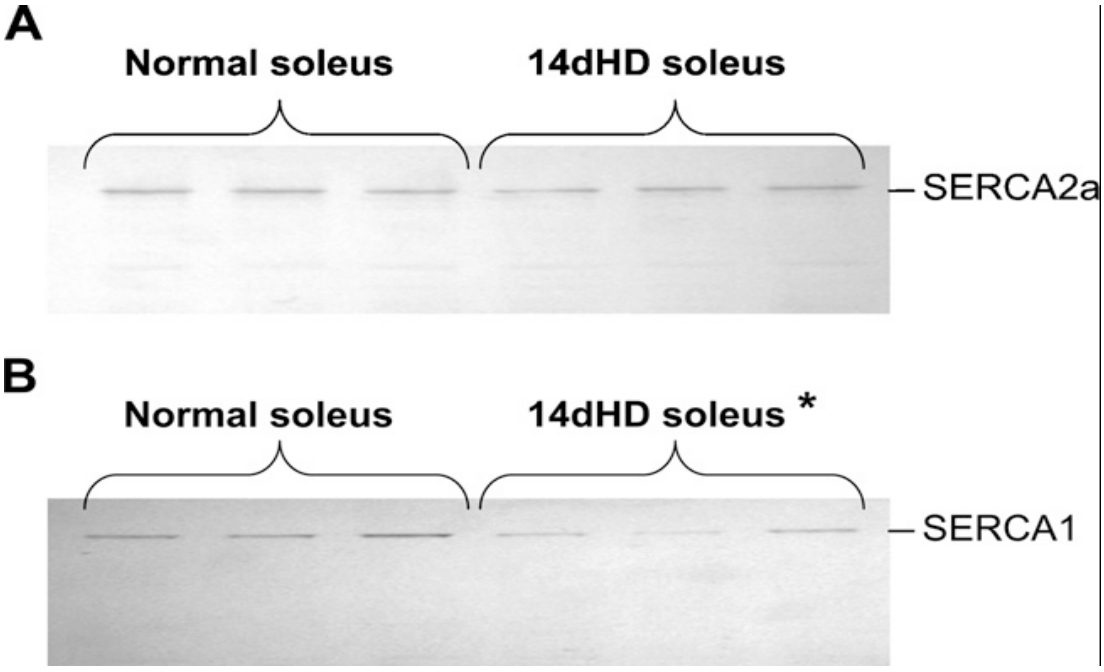


Figure 4: The effect of 14 days hindlimb denervation (14dHD) on SERCA isoforms in soleus of female rats (200g weight). (A) The level of SERCA2a protein showed no difference in the 14-days old hindlimb-denervated muscles compared to the normal while (B) the SERCA1 protein level significantly decreased at day 14 of HD (p=0.0361).

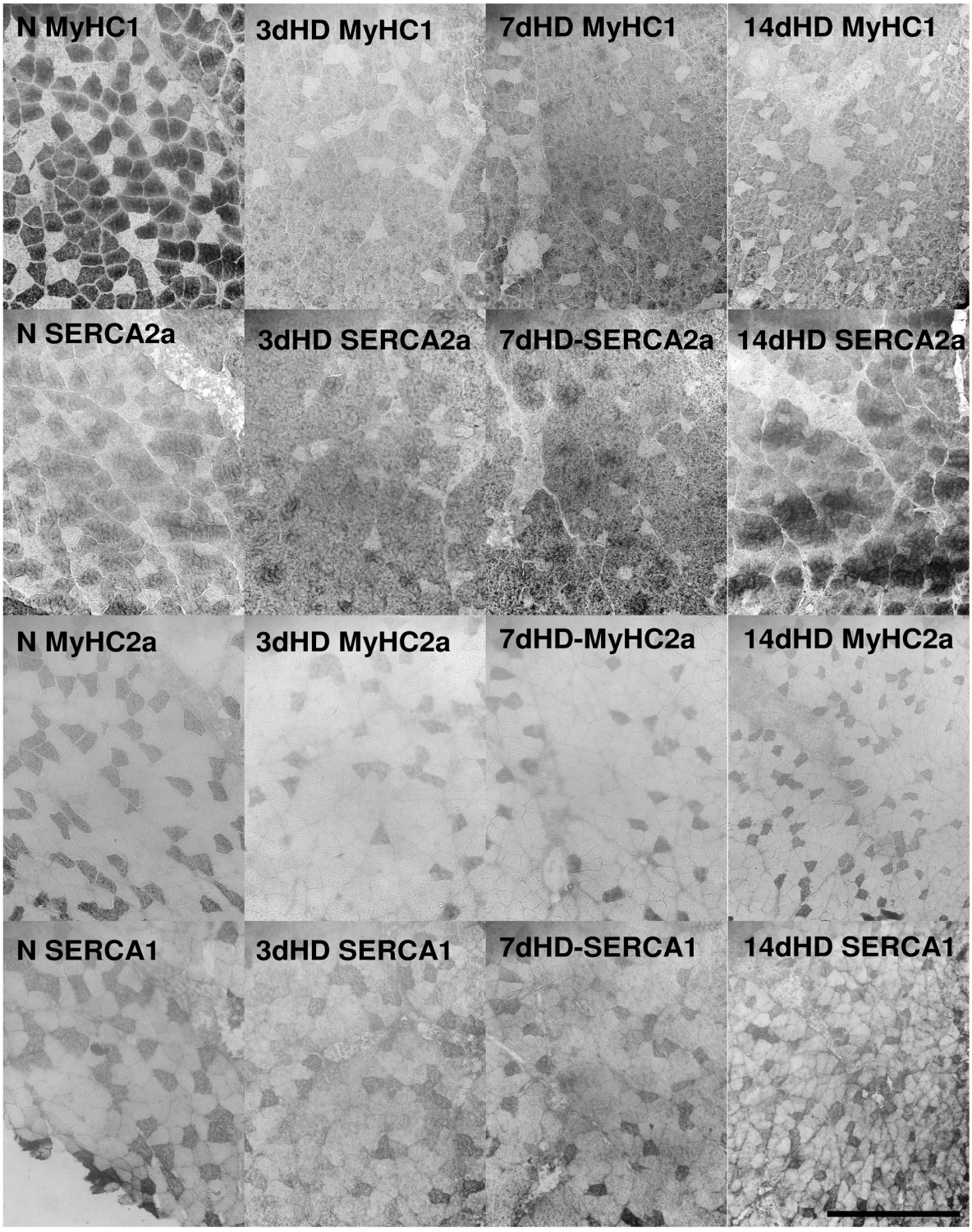

N MyHC1
3dHD MyHC1
7dHD MyHC1
14dHD MyHC1
N SERCA2a
3dHD SERCA2a
7dHD-SERCA2a
14dHD SERCA2a
N MyHC2a
3dHD MyHC2a
7dHD-MyHC2a
14dHD MyHC2a
N SERCA1
3dHD SERCA1
7dHD-SERCA1
14dHD SERCA1

Figure 5A

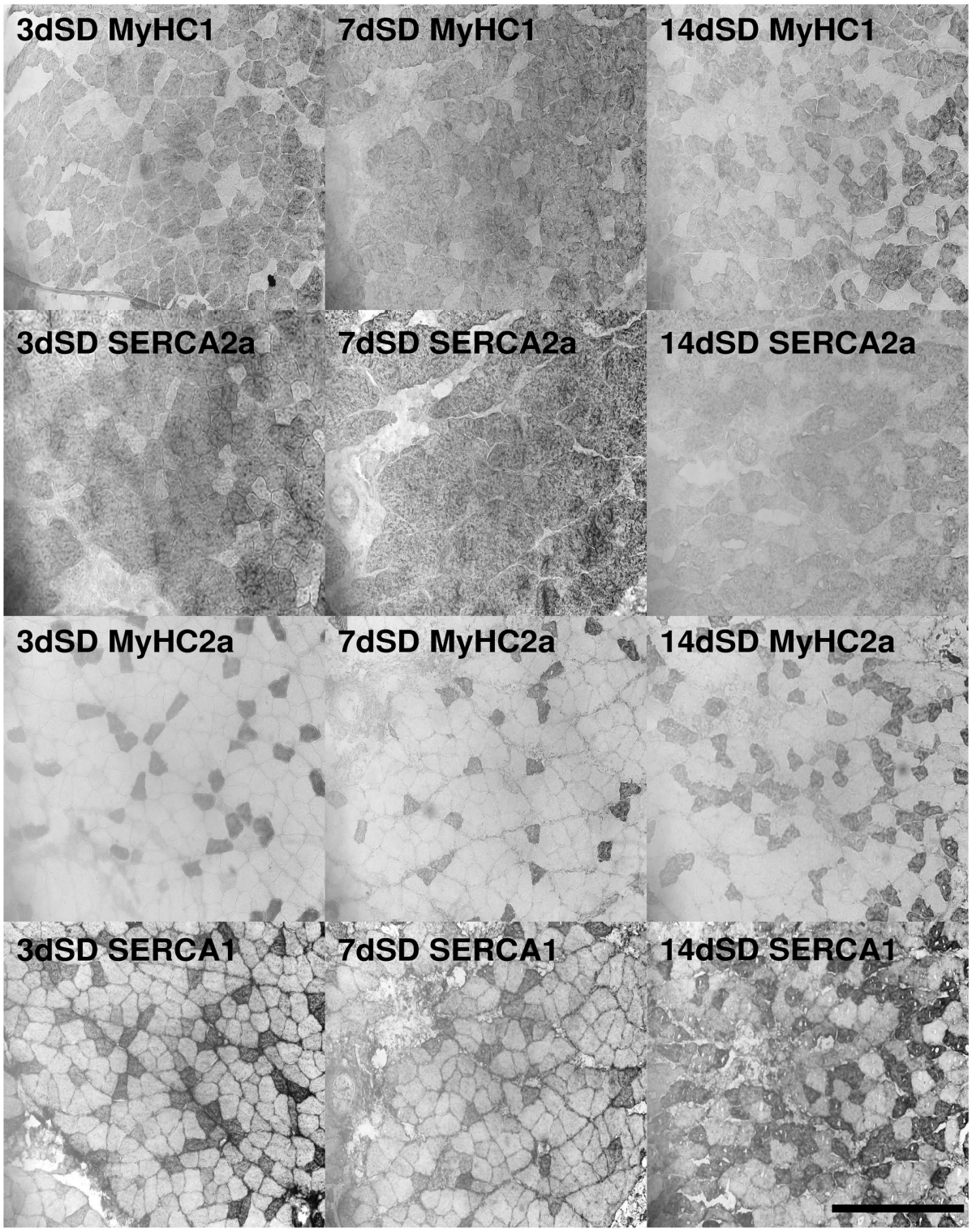

3dSD MyHC1
7dSD MyHC1
14dSD MyHC1
3dSD SERCA2a
7dSD SERCA2a
14dSD SERCA2a
3dSD MyHC2a
7dSD MyHC2a
14dSD MyHC2a
3dSD SERCA1
7dSD SERCA1
14dSD SERCA1

Figure 5B

Figure 5: Fiber distributions of the MyHC and SERCA isoforms in HD and SD. Consecutive parallel sections of (A) control and days 3, 7, 14 HD and (B) days 3, 7, 14 SD soleus muscles are stained for MyHC1, MyHC2a, SERCA2a and SERCA1. Note that majority of fibers co-express MyHC1 with SERCA2a or MyHC2a with SERCA1 however there are more hybrid fibers (which express these proteins in other combinations) in both denervations, particularly in SD. Bars represent 200 μm.

Immunohistochemical results

MyHC fiber types: We counted fibers stained for MyHC1, MyHC2a in both types of denervation. (Fig. 5A, 5B)

The percentage of slow MyHC1-positive type I fibers decreased in all stages of both types of denervation. The decrease was more pronounced in SD than in HD at day 3 (Fig.6A). The percentage of type I fibers was 7.39 % of total fibers lower at day 3 and 3.77 % of total fibers higher at day 7 in SD than in HD muscles. In agreement with this, the decrease of MyHC1 protein level was larger in the SD muscles when compared to the HD ones (Fig. 3A).

In HD muscles the ratio of MyHC2a-expressing type IIA fibers (Fig. 6A) increased on day 7 and 14 in comparison with the normal soleus muscles. However, in SD muscles, the percentage of IIA fibers didn't change compared to normal muscles. On day 7, the ratio of type IIA fibers differed significantly between the HD and SD.

Hybrid fibers: There were more MyHC-hybrid fibers (Fig. 6A) in both types of denervation than in the normal muscles. The numbers of MyHC-hybrid fibers were higher on day 7 in SD soleus muscles than in HD muscles.

SERCA fiber types: Parallel sections were also stained for SERCA1 and SERCA2a. The percentage of pure SERCA2a fibers (Fig. 6B) decreased at day 14 in HD, while in SD muscles it was already lower at day 3 and 7. The SERCA1-positive fibers (Fig. 6B) changed (increased) only in SD at day 7 and 14. Again, the number of SERCA-hybrid fibers increased in both types of denervation, but this was more pronounced in SD muscles (Fig. 6B).

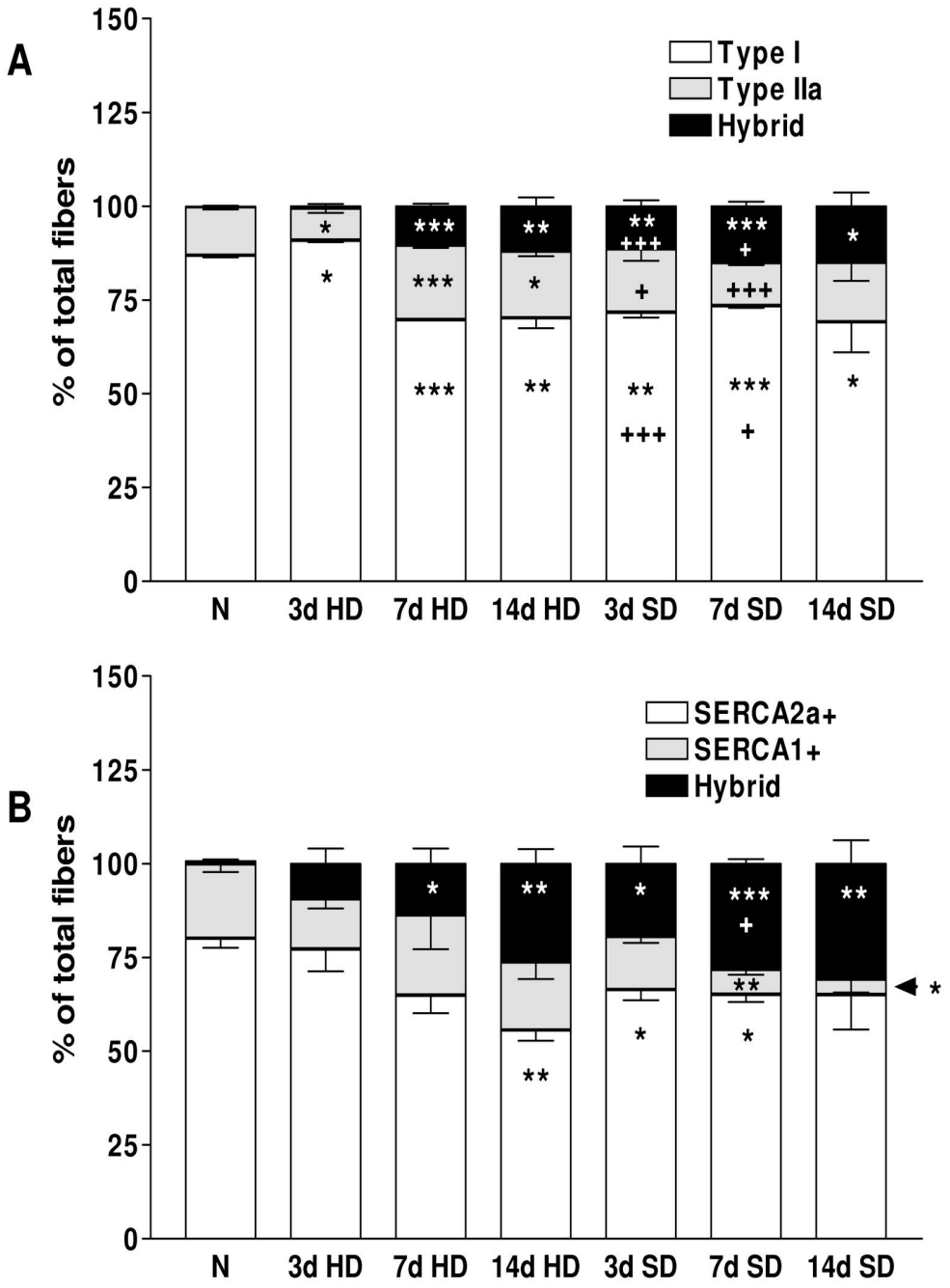
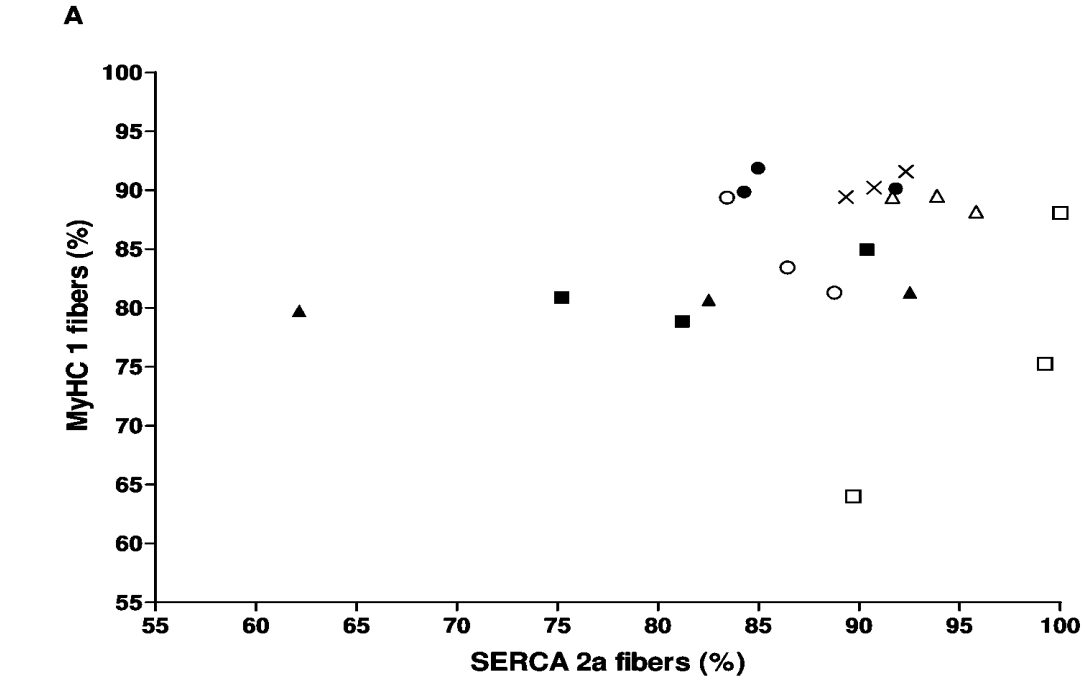

A
% of total fibers
150
125
100
75
50
25
0
Type I
Type IIa
Hybrid
N
3d HD
7d HD
14d HD
3d SD
7d SD
14d SD
B
% of total fibers
SERCA2a+
SERCA1+
Hybrid

Figure 6

Figure 6: The expression of (A) MyHC and (B) SERCA in fibers of denervated soleus muscles. (A) The MyHC expression is shown in fibers in the different stages of both types of denervation. The number of type I (MyHC1) fibers decreases earlier in SD while the number of type IIa (MyHC2a) fibers increases only in HD. The number of hybrid fibers increases more after the first week in SD. (B) SERCA expression is shown in fibers in both types of denervation. The number of SERCA2a-positive fibers decreases earlier in SD but at day 14 this difference disappears. The number of SERCA1-positive fibers decreases only in SD. The number of SERCA-hybrid fibers increases earlier and to a greater extent in SD than in HD. Symbols are as in Fig. 2. An arrow points to the position where the decrease is significant but the symbol does not fit into the bar.

Coexpression of MyHCs with the corresponding SERCAs: Next we explored to what extent the expression of the corresponding slow or fast isoforms of Ca^{2+} pumps and myosins remained correlated during denervation. First we looked at the expressions of the slow isoforms. The correlation between SERCA2a-positive fibers and MyHC1-positive fibers was tight in the normal muscles (r^2=0.987). In HD muscles, it gradually declined (r^2=0.975 at day 3, r^2=0.653 at day 7 and r^2=0.222 at day 14). In SD, after declining (r^2=0.964 at day 3, r^2=0.553 7 day), the correlation totally disappeared at day 14 (r^2=0.00025) (Fig. 7A). Concerning the fast isoforms, the SERCA1-positive and MyHC2a-positive fibers retained a relatively tight correlation during both types of denervation. These fibers didn't show signs of constant decrease of correlation during the two weeks, in contrast to the MyHC1-SERCA2a expressing fibers. (Fig. 7B) (r^2=0.740 in normal muscles; in HD muscles: r^2=0.603 at day 3, r^2=0.875 at day 7 and r^2=0.623 at day 14; in SD muscles: r^2=0.685 at day 3, r^2=0.923 at day 7 and r^2=0.813 at day 14).

Figure 7: Correlation between SERCA and MyHC isoforms in different types of denervations. The results are shown in individual soleus muscles. (A) The percentage of MyHC1-positive fibers and the percentage of SERCA2a-positive fibers on consecutive transversal sections. In normal muscles, the correlation for co-expression of the slow isoforms is strong but it is weaker in HD and SD. The expression of MyHC1 and SERCA2a becomes weakly or uncorrelated in the fibers of HD and SD at day 14. (B) The coexpression of the fast MyHC2a and SERCA1 in fibers is shown. The correlation between MyHC2a and SERCA1 expression in fibers was occasionally weaker in both types of denervations, i.e. at day 3 of HD. X - normal soleus, ● - day 3 of HD, ▲ - day 7 of HD, ■ - day 14 of HD, ○ - day 3 of SD, Δ - day 7 of SD and □ - day 14 of SD.


A
MyHC 1 fibers (%)
SERCA 2a fibers (%)
100
95
90
85
80
75
70
65
60
55
55
60
65
70
75
80
85
90
95
100

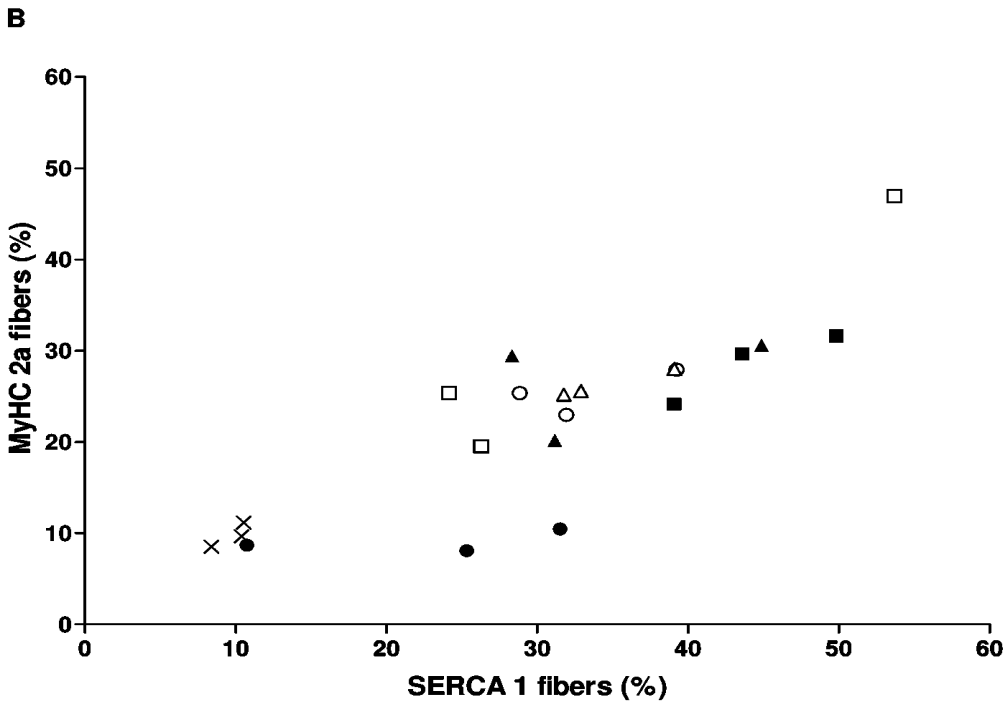

B
MyHC 2a fibers (%)
SERCA 1 fibers (%)
60
50
40
30
20
10
0
0
10
20
30
40
50
60

Figure 7

Diabetic model

Fresh weight, fiber cross-sectional area, total RNA and total protein levels

The weight and protein content of soleus of stz-rats was not significantly different compared to the controls. The fiber CSA of soleus of stz-rats was 83% of that of the control muscles (2912 ± 89 *vs*. 3521 ± 91 μm2, *P*<0.01, n=3)(Table 4).

The mRNA levels

The level of *GAPDH* mRNA was not different in stz-soleus compared to the controls (Fig. 8A), therefore we used it as an RT PCR control, reference mRNA. Normalized levels of *SERCA1*a and *SERCA2a* mRNAs were not significantly lower in the soleus of stz-rats than in the controls (Fig. 8B). Neither were the relative levels of *SERCA1* and *SERCA2* transcripts, measured by ratio-RT PCR, different in the diabetic and control soleus (see right-hand columns in Fig. 8B and representative gel in Fig. 8C). Similarly, there were no significant differences in the *MyHC1*/*MyHC2a* mRNA levels of soleus muscles of the stz-treated and control rats.

Table 4

Morphological parameters of diabetic and control rat soleus muscles. [1]Measured from the first homogenate; [2]two or more adjacent fibers, *P<0.05, **P<0.01, mean ± S.E., n=4 for the weight and protein content and n=3 for the fiber parameters.

Parameter	Diabetic	Control
Weight (mg)	157 ± 9	162 ± 7
Protein content (mg)[1]	34.4 ± 3	31.9 ± 2
Fiber CSA (μm²)	2912 ± 89**	3521 ± 91
Type I fibers (%)	73.5 ± 3.1	80.9 ± 1.6
Type IIA fibers (%)	26.9 ± 3	19.5 ± 1.6
Hybrid fibers (%)	0.5 ± 0.2	0.4 ± 0.1
% of IIA fibers in association[2]	57 ± 4.3*	28.6 ± 5

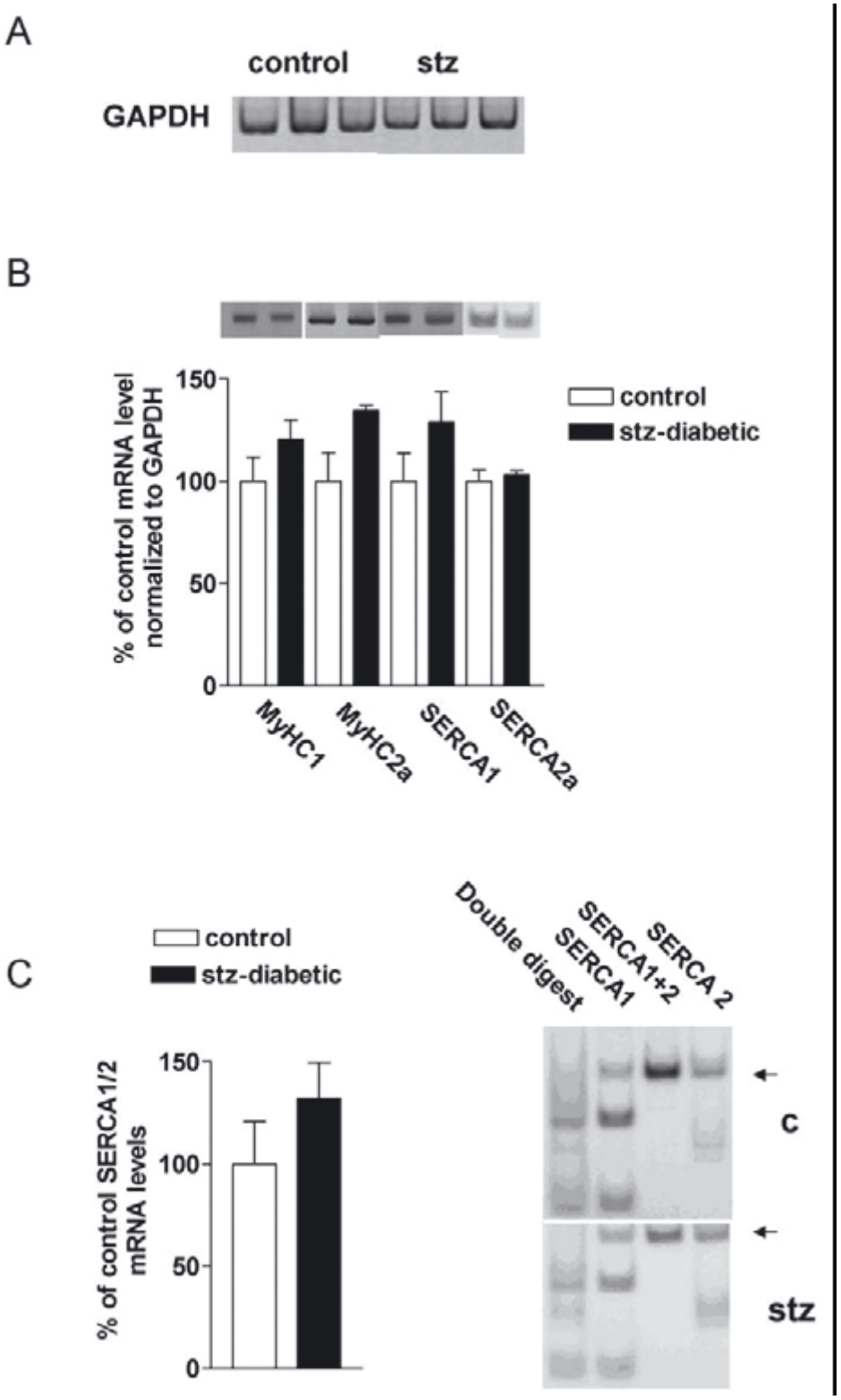

A
control
stz
GAPDH
B
% of control mRNA level normalized to GAPDH
150
100
50
0
control
stz-diabetic
MyHC1
MyHC2a
SERCA1
SERCA2a
C
control
stz-diabetic
% of control SERCA1/2 mRNA levels
150
100
50
0
Double digest
SERCA1
SERCA1+2
SERCA 2
c
stz

Figure 8

Figure 8: Myosin and SERCA mRNA levels in soleus of stz-diabetic rats. (A) GAPDH mRNA levels used for normalization. (B) mRNA levels in percent of control, (mean ± S.E., n=3– 4). Above columns representative bands of RT PCR are shown. (C) The ratio of SERCA1/2 mRNA levels (mean ± SE, n=3 for the control and n=2 for the stz-diabetic rats). Left: Representative gels of ratio-RT PCR analysis of SERCA1 and SERCA2. Undigested SERCA isoforms are indicated above lanes. Only the upper undigested bands (indicated by small arrows) were used for quantitation. c, control; stz, streptozotocin-treated.

The protein levels

In accordance with the mRNA levels, the MyHC1, MyHC2a and SERCA1 protein levels were not different on immunoblots of the soleus of stz-treated and control rats (Fig. 9). However, the SERCA2a level was about 50% lower (P=0.0002) in the soleus of stz-treated rats compared to that of the controls. This was in agreement with the observation made on transverse sections that the SERCA2a level was decreased in type I fibers of the soleus of stz-rats.

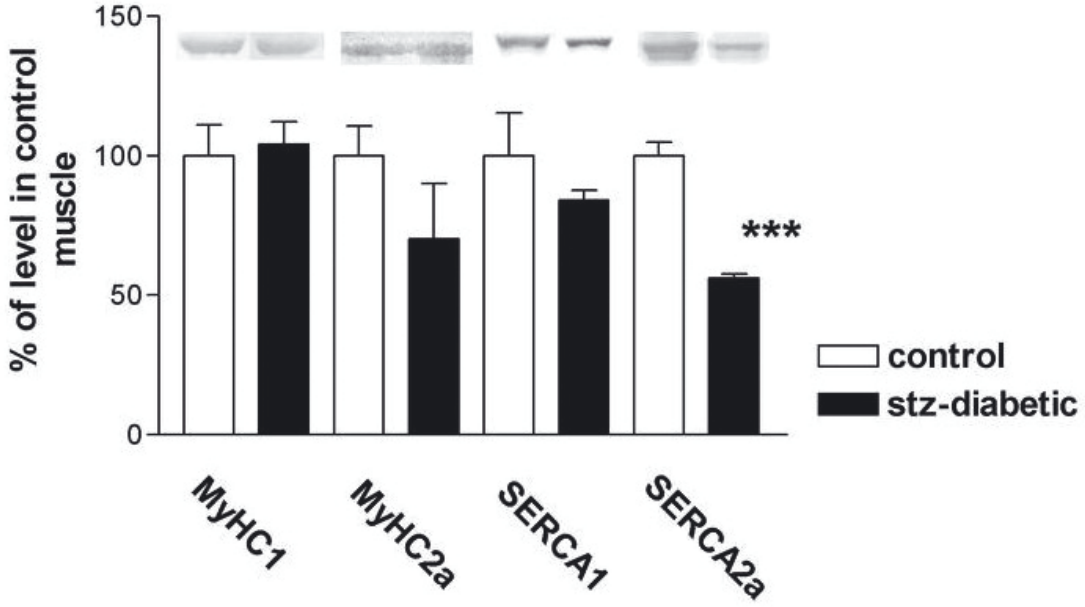


Figure 9: MyHC and SERCA protein levels in soleus of stz-rats. Above columns representative

bands of immunoblots are shown. ***P<0.001, n=3–4. Other symbols are as in Fig. 8.

Immunohistochemical results

The percentage of type I fibers expressing myosin heavy chain (MyHC1) showed a tendency to decline in stz-rats but it was not statistically different from that of the controls (73.5 ± 3.1 *vs*. 80.9 ± 1.6%, P=0.128, n=3). The ratio of type IIA fibers expressing myosin heavy chain 2a (MyHC2a) was also not significantly different in stz-soleus compared to the control (26.9 ± 3% *vs*. 19.5 ± 1.6%, P=0.095, n=3). (Table 4)

The number of hybrid fibers expressing both MyHC1 and MyHC2a was about the same in the soleus of diabetic and controls rats (Table 4). However, in stz-rats the percentage of associated type IIA fibers (two or more fibers together) was higher than in the controls (57 ± 4.3 *vs*. 28.6 ± 5, P<0.05). This indicated peripheral neuropathy and a higher rate of reinnervation in the stz-treated soleus than in the control muscles (Karpati and Engel, 1968; Jaweed *et al.,* 1975).

The MyHC isoforms are the most frequently used but not the only markers of muscle fiber types (Sciaffino and Reggiani, 1996). The sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) isoforms are expressed in coordination with the corresponding MyHCs and can also be used for fiber typing (Talmadge *et al.,* 1996). On consecutive sections we immunostained muscle fibers for SERCA2a, SERCA1, MyHC1 and MyHC2a isoforms (Fig. 10). In adult soleus, SERCA1a practically accounted for the total amount of SERCA1 (Zádor *et al.,* 2007) and it was specifically expressed in the type IIA fibers, whereas SERCA2a was present exclusively in type I fibers. In the diabetic muscles, SERCA1a was stained in type IIA fibers with similar intensity as in the control, but SERCA2a expression in type I fibers was practically not different from the background level found in the type IIA fibers. This was in agreement with the results of immunoblots of the SERCA and MyHC isoforms in the whole soleus muscle.

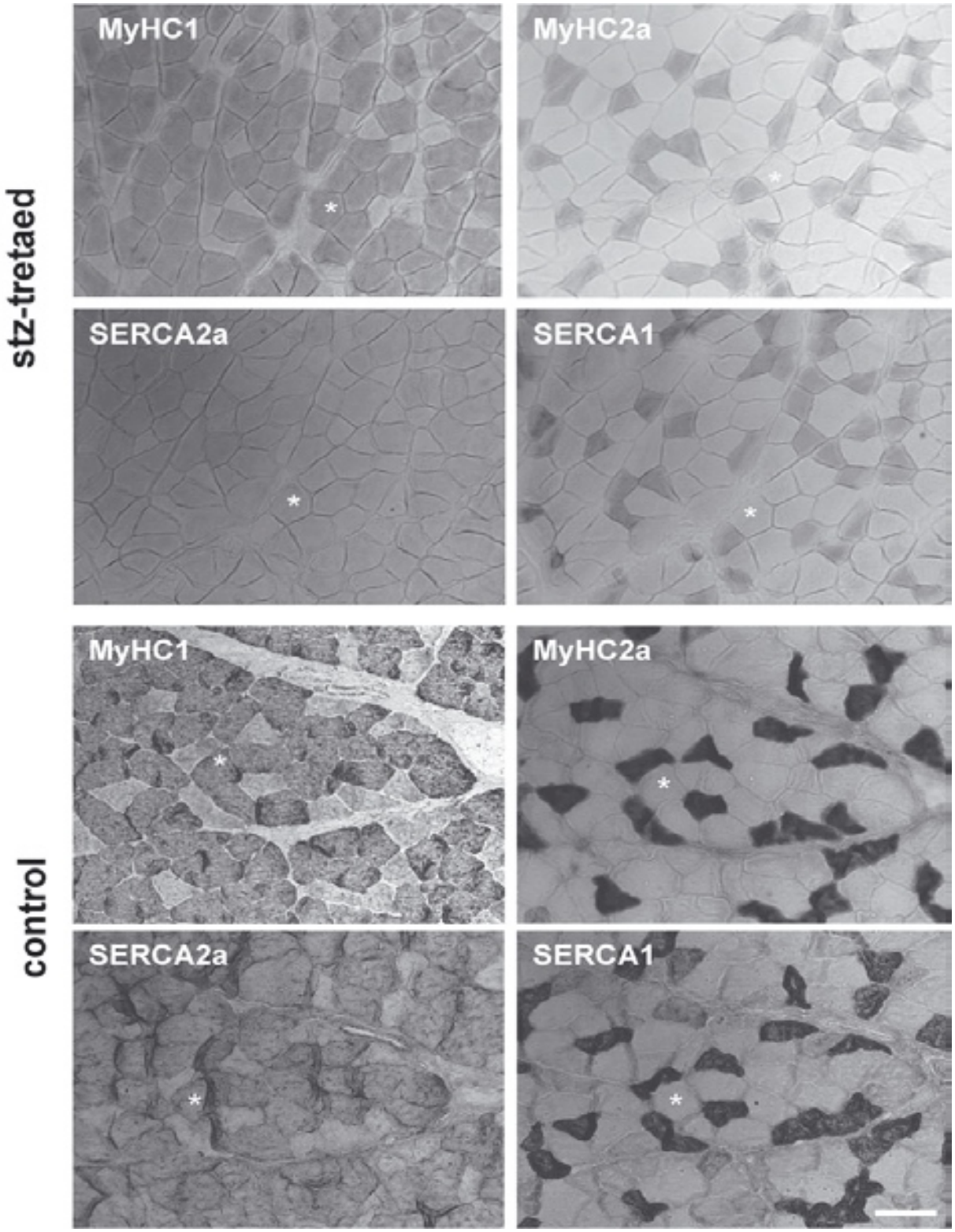

stz-tretaed
MyHC1
MyHC2a
SERCA2a
SERCA1
control
MyHC1
MyHC2a
SERCA2a
SERCA1

Figure 10

Figure 10: Myosin and SERCA isoforms in fibers of soleus muscle of stz-rats. Immunostaining was made on parallel sections. Please note coexpression of MyHC1 with SERCA2a in type I fibers and coexpression of MyHC2a with SERCA1 in type IIA fibers of control and stz-rats. Expression of SERCA2a is practically not distinguishable in type I and type IIA fibers in stz-rats unlike in the control, suggesting lower level of the Ca^{2+} pump. *Indicate identical positions on sections. Bar is 100 μm.

Discussion

Denervation models

We used the methods of SD and HD to highlight the difference in nerve control of expression of MyHC and SERCA isoforms. In SD, the soleus was passively moved by the other hindlimb muscles, in HD, the whole hindlimb was paralysed. In spite of the differences we could observe changes that were common in the both types of denervation. One of these was the switch from slow to fast myosin heavy chains, the other was the less apparent switch from slow to fast SERCA isoforms. This extended the earlier observation of our group that the expression of SERCA isoforms in regenerating muscle are less or not dependent on the nerve compared to the myosin heavy chain isoforms (Zádor and Wuytack, 2003).

It is a novelty that we used SD because others commonly applied the HD method. The less pronounced decrease of fresh muscle mass and fiber CSA in SD compared to HD soleus shows that the denervated muscles are more resistant to general atrophy if they are exposed to passive movement instead of passive state. In disuse, CSA declines together with myofibril number and contractile force (D'Antona *et al.,* 2003) therefore saving CSA is probably benefical.

It is clear that the relative expression levels of slow myosin isoform MyHC1 and slow Ca^{2+} pump SERCA2a are less coordinated in denervated soleus than in the normal muscle. Particularly, the mRNA and protein levels of MyHC1 decreased upon denervation while those of SERCA2a did not change. This shows that SERCA2a is not directly dependent on the nerve in the normal soleus. Our results are at variance with the previous work of Schulte and coworkers (Schulte *et al., 1*994) who described a decline of SERCA2a following hindlimb denervation. However, Schulte *et al. (*1994) used female rats weighing around 200g whereas we used male rats of around 300g. In order to make the comparison more relevant we also

measured the SERCA1 and SERCA2a protein levels in 200g female Wistar rats after 14 days of HD and we obtained similar results as from 300g male rats (Fig. 4). Therefore the difference in the results of Schulte *et al. (*1994) and ours does not result from the sex and age of the rats but more likely from the applied methodology.

For example, Schulte and coworkers (1994) used a cesium chloride gradient sedimentation method and Northern blot to extract and analyse RNA from pooled muscles (4 in case of normal muscles and 6 in case of denervated muscles). Whereas we used the shorter version (Gauthier *et al., 1*997) of the AGPC method (Chomczynski and Sacchi, 1987) and RT-PCR with specific primers in order to detect the mRNA levels in the individual muscles. In our view, our analysis is more adequate to calculate statistical differences in SERCA mRNA levels than those of Schulte *et al. (*1994).

The analysis of SERCA protein levels in Schulte *et al. (*1994) was also different than in our work; they extracted SERCA from microsomes and large membrane fractions. We used the pellet of the first centrifugation for MyHC extraction while the supernatant was further sedimented to obtain the mitochondrial-SR and microsomal fractions (Zádor *et al., 1*998).

Unlike in Schulte *et al.* (1994), we did not relate the amount of SERCA proteins to standards (Kandarian *et al., 1*994). In our work the samples represented the same proportion of each muscle. We chose this method to eliminate one source of errors because the total protein content was more variable in the denervated than in the normal muscle extracts.

The lack of decline in SERCA2a expression (mRNA and protein) that we observed upon denervation is in agreement with the steady SERCA2a levels in spinal cord isolation (Zhong *et al.,* 2002), in spinal cord transsection (Talmadge and Paalani, 2007), in regenerating-denervated muscles (Zádor and Wuytack, 2003; Zádor *et al.,* 2005) and in overloaded soleus muscles (Awede *et al.,* 1999).

In contrast to SERCA2a, the level of SERCA1 clearly declined in the soleus after two weeks of denervation. This means that the changes in the level of the fast muscle specific Ca^{2+} pump were also not coordinated with those of the fast-type MyHC2a, since the latter remained unchanged upon denervation. However, at the level of the fibers the coordination of expression of SERCA1 and MyHC2a was stronger than that of SERCA2a and MyHC1.

It is worth to note that independently from the protein, both SERCA1 mRNA isoforms were increased at 3 days of denervation. A similar increase of these mRNAs can be induced

by stretch (Zádor *et al.,* 1999; 2007). SD increased the mRNA level of both SERCA and MyHC isoforms compared to HD. This implies that there exist layers of regulation of gene expression at the mRNA and the protein level that might act differently and independently (Leberer *et al., 1*986; Huey and Bodine, 1998).

The largest discrepancy in coordinated protein expression was found when upon SD the MyHC1 protein level dramatically decreased whereas the SERCA2a did not change. This drop in the level of MyHC1 protein was more than could be expected from a lack of maintenance by innervation: it indicated an adaptation to passive movement (similar to the mild transient increase of total RNA and protein content). A parallel drop in the level of SERCA2a was not observed, again showing that myosin and Ca^{2+} pumps respond differently to passive movement.

Upon denervation, the number of MyHC1-positive and SERCA2a-positive fibers decreased more or less together, but, for both types of denervation, after 14 days the coordination in expression of these isoforms was practically lost. The loss of coordination was more pronounced in SD. A similar, but much less pronounced loss of coordination was observed in both denervations for the fast isoforms: MyHC2a and SERCA1. The loss of coordinated expression resulted in the appearance of hybrid fibers. The ratio of MyHC1-MyHC2a hybrid fibers, SERCA1-SERCA2a hybrid fibers (Fig. 6) and mismatch fibers (expressing MyHC1, SERCA1, MyHC2a and SERCA2a) increased after 7 or 14 days in both type of denervations. More SERCA-hybrid fibers than MyHC-hybrid fibers were found in SD than HD and the SERCA-hybrids appeared earlier. This also shows that the expressions of MyHC and of SERCA are differentially regulated.

At longer term (*i.e.* denervation of more than 72 days) the co-expression of the corresponding myosin and SERCA isoforms again match (Hämäläinen and Pette, 2001). A likely explanation for this is that the general atrophy reduces both MyHC1 and SERCA2a level with a different time-course. Another explanation could be that the structure of the sarcoplasmic reticulum, that houses the SERCA, is nerve-activity dependent (Heck and Davis, 1988; Salvatori *et al., 1*988), and thereby indirectly affects the expression/stability of SERCA2a. The regulation of gene expression is also likely to be different for the myosin heavy chains and SERCAs. I.e. the slow myosin promoter is activated by MEF-2D, MyoD, p300, and the calcineurin/NFATc1 pathway (Meissner *et al., 2*007), similar but different factors (MEF-

2C, NFATc4) are known to upregulate the SERCA2a promoter (Vlasblom *et al., 2*004). In addition, in regenerating muscle the MyHC1 protein mostly follows its transcript level while the SERCA2a is largely controlled posttranscriptionally (Zádor and Wuytack, 2003).

In conclusion, the expression of SERCA and myosin isoforms seems to be separately controlled each by a unique set of regulatory factors in the soleus muscle. The slow innervation is a major controller for the slow type myosin, while the fast myosin (MyHC2a) is more expressed when the slow myosin is declining. The vanishing of the slow myosin isoform upon denervation is however not balanced by an equimolar replacement of the fast isoform. Therefore the switch of SERCA2a to SERCA1a isoform does not coincide, but is rather delayed compared to the slow-to-fast switch of the myosin isoforms. As a consequence, the slow type SERCA is not expressed in coordination with the slow type myosin in many of the fibers and, partially because of this, there are more SERCA-hybrid fibers than myosin-hybrid fibers.

Diabetic model

The soleus muscle of stz-rats showed similar weight, protein content but a smaller CSA than the control. This suggests that fiber size reflects muscle atrophy earlier than the fresh weight or protein content.

An increased association of type IIA (fast oxidative) fibers is an indication of peripheral neuropathy (Karpati and Engel, 1968, Jaweed *et al.,* 1975), and it also appears to be an early marker of diabetes in stz-soleus (Snow *et al.,* 2005). Parallel with fiber type association, we detected a tendency but not a significant transition of slow to fast fiber types at 4 weeks after stz treatment. We used only three animals for this experiment, but increasing the sample size theoretically above n=14 in both groups should result in a significant difference of fiber types according to a power analysis of our data. This was in agreement with Rutschmann *et al. (*1984) who also reported no decline of slow myosin level after the same duration (4 weeks) in stz-rats, in contrast to reports of lower slow myosin levels and significant slow-to-fast fiber type transition at 2–3 months after stz treatment (Snow *et al.,* 2005; Punkt *et al.,* 1999). The above studies all used n≤8 rats per group. The range of streptozotocin doses used for inducing type I diabetes in rat is relative narrow in rats (reviewed by Szkudelski, 2001), therefore the variance in the fiber type shift is more likely due to the duration of hyperglycemia than to the

applied amount of the toxin.

According to the unchanged ratio of myosin and SERCA1a in fiber types, the stz-soleus showed similar MyHC1, MyHC2a and SERCA1a mRNA and protein levels compared to those of the control. The lower expression level of SERCA2a in diabetic soleus type I fibers was supported by the immunoblotting data but not the RT PCR results. This shows that different mechanisms regulate the levels of the SERCA2a pump and the slow myosin in the stz-soleus.

The SERCA2a protein level decrease in several ways (reviewed by Vangheluwe *et al.,* 2005). In diabetic rat heart, it may declines because of an increased level of glycolysation (Bidasee *et al.,* 2004). Advanced glycation end products also accumulate in the skeletal muscle of diabetic animals (Snow *et al.,* 2006). Although the levels of both SERCA2a and its inhibitor phospholamban decrease in diabetes, the phospholamban/SERCA2a ratio becomes higher (Rupp *et al.,* 1989; Belke *et al.,* 2004; Bidasee *et al.,* 2004; Vasanji *et al.,* 2004). The levels of reactive oxygen and nitrogen species (ROS and RNS) are elevated (Aragno *et al.,* 2004; Mastrocola *et al.,* 2008) and they decrease the levels of specific proteins like SERCA2a (Aragno *et al.,* 2004; Mastrocola *et al.,* 2008, reviewed by Vangheluwe *et al.,* 2005).

Both SERCA1 and SERCA2 isoforms interact with phosphorylated forms of insulin receptor substrates, IRS-1 and IRS-2 (Algenstaedt *et al.,* 1997). This interaction can be stimulated by insulin in the control but not in stz-rats (Algenstaedt *et al.,* 1997). It is an interesting question whether the decreased interaction of IRS and SERCA in stz-diabetic rats (Algenstaedt *et al.,* 1997) is related to the decline of SERCA2a level reported here.

The pathological mechanism of diabetes involves inhibition of phosphatidylinositol 3-kinase (PI3-K) (Lee *et al.,* 2004). Low activity of the PI3-K/Akt pathway in the endothelium and/ or skeletal muscle may contribute to defective signals and subsequent catabolism (reviewed by Kobayashi *et al.,* 2005). However, a direct role of this pathway in the phosphorylation of SERCA2a or its regulator, phospholamban, has not been documented yet.

The expression of SERCA2 isoforms is largely regulated at the level of RNA splicing in the normal heart and slow skeletal muscle, i.e. the structure of the 3' end confers higher stability on *SERCA2a* than on *SERCA2b* mRNA (reviewed by Misquita *et al.,* 2006). However, no such mechanism was implicated in the diabetic soleus (in our experiments) or in the diabetic heart (Bidasee *et al.,* 2004), since the SERCA2a mRNA level was not different from that of

the control. The splicing of SERCA1, SERCA2 and the muscle-specific insulin receptor are each regulated by muscleblind protein 1 (MLNB1) (Savkur *et al.,* 2001; Hino *et al.,* 2007; Ho *et al.,* 2004) but no such regulation was implicated in the stz-soleus.

The calcineurin-NFAT pathway is a key factor of muscle remodeling (reviewed by Bassel-Duby and Olson, 2006) and it has been reported to decline in stz-induced diabetic rats parallel with muscle atrophy (Costelli *et al.,* 2007). Conclusively, muscle specific overexpression of calcineurin improves insulin action and glucose metabolism in the soleus of transgenic mice (Ryder *et al.,* 2003; Long *et al.,* 2007). Since SERCA2a is the major pump regulating the level of Ca^{2+} in the sarcoplasm, it is an interesting question how the decrease of SERCA2a level reported here is related to the decline of the calcineurin-NFAT pathway in diabetic muscle.

Our results suggest that the SERCA2a protein level is an earlier marker than that of MyHC1 for muscle diabetes. This implicates that the Ca^{2+} metabolism reacts more readily to diabetic stress than the contractile elements do. The separate regulation of SERCA2a and MyHC1 is a further support of this conjecture.

Comparison of levels of SERCA and MyHC in denervation and diabetes

The two denervation models and the diabetic model are different in many aspects, but also show a number of similarities. The two denervation models are examples of the effects of acute lack of innervation while the diabetic model represents the effects of a chronic metabolic malfunction. These different pathological statuses explain the differences between the patterns of changes in the two models. I.e. the unchanged weight and the decrease of fibers size in the diabetic soleus is similar to the results from selectively denervated soleus, where the decrease in fiber size but not in fresh weight or protein content has been reported after 3 days, while both fresh weight and protein content decreased with fibers size after 7 days. This shows that fibers size adapts earlier than fresh weight and represents an example of the partial overlap between the effects of acute and chronic conditions.

During denervation the MyHC1 decreases quickly in the soleus muscle fibers, showing that this protein is highly dependent on slow-type innervation. This change is followed with a large lag by the slow-type SERCA2a, showing that this protein is not as strongly dependent on innervation as MyHC1.

The changes of these proteins are in contrast with the results in the hyperglycaemic soleus. In this model the SERCA2a protein was the first to decrease and followed only much later by the decrease of MyHC1. This shows that different mechanisms regulate the levels of the SERCA2a pump and the slow myosin in diabetic than in denervated soleus, because, in the latter one, the dysregulation occurred both at the mRNA and protein levels

Although the detailed patterns of changes differ from each other in the three models, the directions of changes point to slow-to-fast transformation and reveal discoordination in all. Overall, these elements are underlining the different paths of regulation of corresponding MyHC and SERCA isoforms, showing that the MyHC1 is more dependent on innervation while SERCA2a is more dependent on metabolic changes.

The novel results in the thesis:

- The regulation of the corresponding MyHC and SERCA isoforms dyscoordinated in denervation, among these the MyHC1 is dependent as long as the SERCA2a is not dependent on innervation.
- The selective denervation is introduced as a new experimental model to study gene expression.
- In the stz-induced diabetes the slow-type SERCA2a is more dependent on metabolic status than the slow-type MyHC1.
- The decline of fibers size (CSA) reflects muscle atrophy earlier than that of the fresh weight.

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Annex

Papers related to the Thesis