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DIFFERENTIAL EXPRESSION OF GENES INVOLVED IN THE CALCIUM HOMEOSTASIS IN MASTICATORY MUSCLES OF MDX MICE

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Duchenne Muscular Dystrophy (DMD) and its murine model, mdx, are characterized by Ca^{2+} induced muscle damage and muscle weakness followed by distorted dentofacial morphology. In both, DMD patients and in mdx mice, could be proven so far that only the extraocular muscles (EOM) are not affected by muscular dystrophy. The EOMs are protected against calcium overload by enhanced expression of genes involved in the Ca^{2+} homeostasis. We could recently demonstrate that masticatory muscles of mdx mice are differentially affected by muscle dystrophy. The dystrophic masseter and temporalis shows muscle histology comparable to all other skeletal muscles in this animal model, whereas dystrophic tongue muscles seem to develop a milder phenotype. Due to this fact it is to hypothesize that an altered Ca^{2+} homeostasis seems to underlie the mdx masticatory muscle pathology. Aim of this study was to examine the mRNA and protein levels of the sarcoplasmic reticulum Ca^{2+}ATPases SERCA1 and SERCA2, the plasma membrane Ca^{2+}ATPases Atp2b1 and Atp2b4, the sodium/calcium exchanger NCX1, the ryanodine receptor 1, parvalbumin, sarcolipin, phospholamban and the L-type Ca^{2+} channel alpha-1 subunit (Cacna1s) in Musculus masseter, temporalis, and tongue of 100 day old control and mdx mice. In mdx masseter muscle significant increased mRNA levels of NCX1 and Cacna1s were found compared to control mice. In contrast, the mRNA amount of RYR1 was significant reduced in mdx temporalis muscle, whereas ATP2b4 was significant increased. In mdx tongue a down-regulation of the ATP2b1, sarcolipin and parvalbumin mRNA expression was found, whereas the phospholamban mRNA level was significantly increased compared to controls. These data were verified by western blot analyses. Our findings revealed that mdx masticatory muscles showed an unequally altered expression of genes involved in the Ca^{2+} homeostasis that can support the differences in masticatory muscles response to dystrophin deficiency.

Key words: *Duchenne muscular dystrophy, calcium homeostasis, masticatory muscles, differential expression, mdx mice, calcium ATPase, sodium/calcium exchanger*

INTRODUCTION

A hereditary severe muscles disease, Duchenne muscular dystrophy (DMD), and its animal model, the mdx mice (X-linked muscular dystrophy), lead to progressive fibrosis and weakness of muscular tissue. This chronic ailment is caused by mutation in the gene dystrophin, a cytoskeletal protein which contributes to the stability of muscle cell membranes and is essential for the long-term function of skeletal muscle (1). The DMD mainly results in respiratory failure and in dilated cardiac myopathy (2). However, the outcome of DMD is also reflected in orofacial system where affected masticatory muscles often have effects on the occlusion (3). Dysfunction of the masticatory muscles usually leads to various diseases of the stomatognathic system, such as pain in the ear region, impairment of the course of abduction and adduction of the mandible, lack of symmetry of its lateral movements and excessive tension of stomatognathic system's muscles (4). In various studies a high prevalence of malocclusions and orofacial dysfunctions was found in DMD patients, including posterior cross bites, anterior and lateral open bites and skeletal Class III malocclusion with dental compensation (5-9).

Muscle activity and function appear to be related to ionic concentrations in the muscle (10). Cardiac and vascular contraction is initiated by calcium *via* influx in cardiac and smooth muscle from extracellular fluid (11). This fact could also explain why there is a mechano-sensitive response of the vascular system during mechanical stimulation of the masseter muscle (12). The muscle plasticity is closely linked to and highly dependent on the Ca^{2+} handling system. The regulation of Ca^{2+} homeostasis beside the Ca^{2+} pumps of the plasma membranes such the plasma membrane Ca^{2+}-ATPase (PMCA), the Na^{+}/Ca^{2+} exchanger (NCX1), or the Ca^{2+} pump of sarco(endo)plasmic reticulum (SERCA), it also involves a large array of Ca^{2+} binding proteins, for instance troponin and parvalbumin (13), and other proteins like sarcolipin (SLN) and phospholamban (PLN) which are confined to the ER or SR and inhibit the SERCA activity by lowering the apparent Ca^{2+} affinity of the pump (14, 15). One other protein located in the SR, the ryanodine receptor (RyR) releases Ca^{2+} in the cytostol (16).

The absence of dystrophin in muscles can result in abnormally regulation of sarcolemmal channels responsible for calcium handling (2). Numerous studies on dystrophic muscles have shown that the dystrophin-deficient muscle fibres are overloaded with calcium and the increased protein degradation results from the elevated Ca^{2+} levels (1). Moreover, there is unquestionable evidence that voltage-independent calcium leak channels and stretch-activated calcium channels show an increased activity in dystrophic muscle cells (17). Another potential reason giving rise to calcium accumulation is an incorrect function of L-type Ca^{2+} channels (18). Defects in the Ca^{2+} regulating proteins might also result in abnormal chronic and acute Ca^{2+} concentrations which can lead to various forms of impaired muscle function (16).

In a previous study was shown, that extraocular muscles (EOMs) of both, DMD patients and dystrophic mice, are not clinical and histological affected by muscular dystrophy (19). The EOMs are protected against calcium overload by an enhanced expression of genes involved in the Ca^{2+} homeostasis (20). Recently, it was demonstrated that different dystrophic masticatory muscles develop inter-muscular differences in muscle histology and gene expression pattern in response to dystrophy. The dystrophic masseter and temporalis shows muscle histology comparable to all other skeletal muscles as found in mdx mice, whereas dystrophic tongue muscles seem to develop a milder phenotype (21-24). Following the hypothesis that an altered Ca^{2+} homeostasis seems to underlie the mdx masticatory muscle pathology, the aim of the current study was to investigate changes of the expression level of proteins involved in Ca^{2+} handling, in order to explain the inter-muscular differences. The investigation of pathological events in masticatory muscle atrophy is urgently needed to understand the disease more profoundly and develop therapeutic strategies for DMD patients.

MATERIAL AND METHODS

Animals

Mice of the inbred strains C57Bl/10ScSn (control) and C57/Bl10ScSn-*Dmd*^{mdx}/J (mdx) were obtained from the Department of Orthodontics from the University Medicine of Greifswald. Age-matched pairs of mdx and control animals (each n = 9–13; 100 d) of either sex were killed using isofluran inhalation. All procedures were approved by a governmental committee on animal welfare on the State Government (LALLF M-V/TSD/7221.3-2.3-001/09). The muscle tissue samples were prepared from the superficial part of masseter, the middle of the temporal muscle and the superior longitudinal tongue muscle and shock-frozen in liquid nitrogen for quantitative reverse transcription PCR and Western blot analysis.

RNA extraction and reverse transcription

Total RNA was isolated using guanidinium-isothiocyanate (RNeasy Fibrous Mini Kit, Qiagen, Hilden, Germany) and RNA concentration was determined by UV absorbance measurements. An amount of 200 ng total RNA was transcribed reversely using random hexamer primers and the TaqMan Reverse Transcription Reagents (PE Applied Biosystems, Weiterstadt, Germany) as described previously (21, 22, 24-37).

TaqMan RT-PCR

The method was performed as described previously (21, 22, 24, 25) using a real-time PCR cycler (StepOne Plus, Applied Biosystems). Gene specific primers and probes were purchased from PE Applied Biosystems (*Table 1*) with each probe having been synthesized with a fluorescent 5´-reporter dye (FAM: 6-carboxy-fluorescein) and a 3´-quencher dye (TAMRA: 6-carboxy-tetramethyl-rhodamine). All values are given in relation to the mRNA of 18S rRNA. A "no-template control" using water was performed parallelly in all experiments. Each series of experiments was performed twice.

Western blot analysis

Muscle tissue samples were mechanical homogenized during thawing in lysis buffer (5% glycerol, 0.1% Triton X-100) supplemented with a protease inhibitor cocktail (Sigma, Taufkirchen, Germany) using the SpeedMill P12 homogenizer (Analytikjena, Jena, Germany). The total protein extracts (30 µg) were separated on SDS gels, transferred to nitrocellulose membranes (Schleicher & Schuell) using a tank blotting system (Gibco-BRL, Germany) and incubated with the primary antibody (*Table 2*) over night at 4°C. Subsequently, HRP-conjugated goat anti-rabbit or goat anti-mouse immunoglobulins (DAKO, Hamburg, Germany) were used at a dilution of 1:5000. Visualisation and detection of bound antibodies were carried out using an enhanced chemiluminescence system (Perbio Science). To assess equal loading of the gel, every membrane was stripped with Restore™ Plus Western blot stripping buffer (Perbio Science) and incubated with a monoclonal anti-α-actinin antibody (clone AT6/172, Upstate, dilution 1:1000; two hours at room temperature) or monoclonal anti-actin antibody (clone C4,

Table 1. TaqMan primer and probes purchased by Applied Biosystems.

Assay name	Accession number of the gene	Assay number
SERCA1	NM_007504.2	Mm01275321_m1
SERCA2	NM_001110139.2	Rn01499542_m1
Ryr1	NM_009109.2	Mm01175172_g1
Slc8a1 (NCX1)	NM_019268.2	Rn01472835_m1
ATP2b1 (ATPase 1)	NM_026482.2	Mm01245810_m1
ATP2b4 (ATPase 4)	NM_001167949.1	Mm01285602_m1
Pvalb	NM_013645.3	Mm01195641_m1
Sln	NM_025540.2	Mm00481536_m1
Pln	NM_022707.1	Rn01434045_m1
Cacna1s	NM_001081023.1	Mm01285322_m1

Table 2. Details and incubation protocols of the used antibodies.

Antibody	Isotype	Producer	Incubationprotocol
Parvalbumin alpha	sheep polyclonal	Lifespan Biosciences (Hamburg, Germany)	1:750 in PBS containing 5% powdered milk and 0.025% NaN_3
SERCA1	mouse monoclonal	Dianova (Hamburg, Germany)	1:500 in PBS containing 5% powdered milk and 0.025% NaN_3
SERCA2	rabbit polyclonal	Abcam (Cambridge, United Kingdom)	1:1500 in PBS containing 5% powdered milk and 0.025 % NaN_3
phospholamban	rabbit polyclonal	Abcam (Cambridge, United Kingdom)	1:500 in PBS containing 3% BSA and 0.025% NaN_3
NCX1	mouse monoclonal	Abcam (Cambridge, United Kingdom)	1:200 in PBS containing 5% powdered milk and 0.025% NaN_3

Table 3. Gene-specific transcript levels in masticatory muscles of 100 day old control and mdx mice. The mRNA levels are given in relation to the 18S rRNA. Means ±S.E.M. are given in all cases for n = 9–13 samples. Bold and italic numbers indicate significant differences between control and mdx mice, Student´s t-test with degrees of freedoms in parentheses or Mann-Whithney-U rank sum test. n.s. = not significant.

Gene name	Tongue		Masseter		Temporalis		P value
	control	mdx	control	mdx	control	mdx	
Ryr1	3034 ±476	2382 ±280	2335 ±530	4789 ±1417	**14669 ±4618**	**4508 ±258**	P=0.006
SERCA1	21121 ±2152	17133 ±1751	20711 ±6957	26792 ±5084	57334 ±5793	44885 ±4947	n.s.
SERCA2	38±6	35±5	21±10	14±3	16±3	17±10	n.s.
Slc8a1	8.1±2.3	4.6±0.7	**2.7±0.4**	**42.9 ±18.1**	14.7±5.0	24.4 ±11.2	P=0.024
ATP2b1	**177±28**	**105±14**	46±9	26.5±10	110±21	77±11	P=0.043 (19)
ATP2b4	603±73	501±54	55±15	27±7	**75±18**	**127±14**	P=0.04 (16)
Pvalb	**2118 ±260**	**911 ±112**	27550 ±6243	21652 ±3088	63595 ±18413	38049 ±8120	P=0.0002
Sln	**26558 ±7658**	**9105 ±1431**	105±32	104±16	212±53	373±111	P=0.001
Pln	**0.19 ±0.12**	**0.36 ±0.18**	0.07 ±0.04	0.17 ±0.09	0.09±0.09	0.27±0.18	P=0.048 (16)
Cacna1s	1176 ±298	750 ±92	**547±98**	**1480 ±417**	2754 ±647	2182±448	P=0.013

Millipore, dilution 1:1000; two hours at room temperature). Quantitative analyses of protein bands from parvalbumin, SERCA1, SERCA2, phospholamban, NCX1, actin and α-actinin in masticatory muscles were carried out using the GelScan 5.2 software (Serva, Germany). Mod (mean optical density) ±S.D. are given in all cases for n=3 independent Western blot analyses (muscle probes from three different mice of each mice strain). All values are shown in relation to values of actin or α-actinin, respectively.

Statistical analysis

For gene expression the obtained values were compared using Student's unpaired t-test. If the normality test failed the Mann-Whithney-U rank sum test was used. In contrast, for protein expression analyses the obtained values were compared using paired t-test. If normality test failed the Wilcoxon signed Rank test was used. $P<0.05$ was considered as statistically significant. Statistical analysis was performed using the SigmaStat version 3.5 software (Systat Software, San Jose, USA). All gene expression data are given as means ±S.E.M., whereas protein expression data are given as means ±S.D.

RESULTS

TaqMan RT-PCR

Quantitative TaqMan RT-PCR performed on samples of masticatory muscles revealed the presence of all tested genes in masseter, temporalis and tongue muscular tissue. The gene expression of NCX1 and $Ca_v1.1$ was increased 15.8 fold and 2.7 fold in mdx masseter muscular tissue when compared to the control, respectively (*Table 3*). In contrast, the mRNA amount of

RYR1 was significantly reduced in mdx temporalis muscle (MW ±S.E.M.; control versus mdx: 14669±4618 versus 4508±258, P=0.006), whereas ATP2b4 was significantly increased (75±18 versus 127±14; P=0.04; *Table 3*). Most differences in the gene expression between control and mdx mice were found in tongue muscles. In mdx tongue a down-regulation of about 40%, 65% and 57% of the ATP2b1, sarcolipin and parvalbumin mRNA expression was found, respectively. Furthermore, the phospholamban mRNA level was significantly increased compared to controls (0.19±0.12 versus 0.36±0.18; P=0.048; *Table 3*).

Western blot analysis

Western blot analysis allowed us to semi-quantify SERCA1, SERCA2, NCX1, phospholamban and parvalbumin proteins (*Fig. 1*). Single immunoreactive bands of approximately 110, 115, 100, 6 and 12 kDa, were detected, respectively. These bands agree with the expected molecular weights of these proteins (*Fig. 1*). The quantitative evaluation of the Western blots did not reveal any differences in the expression of SERCA1 and SERCA2 between mdx and control mice (*Fig. 2A*, *2B*). The parvalbumin western blots showed significantly decreased levels between mdx and control mice in tongue and masseter muscles (mean ±S.D.; control versus mdx: tongue, 2.38±1.1 versus 1.6±0.9, P=0.031; masseter, 3.17±0.85 versus 2.48±1.18, P=0.031). A reduction of 22% and 33% in protein expression in dystrophic muscles was detected, respectively (*Fig. 2C*). The protein expression of phospholamban was significantly increased only in dystrophic masseter muscle (mean ±S.D.; control versus mdx: masseter, 0.96±0.24 versus 1.35±0.31, P=0.046 with 4 degrees of freedom, *Fig. 3A*). Furthermore, in dystrophic masseter muscle a significantly higher NCX1 protein expression was detected compared to control muscles (masseter, 0.49±0.05 versus 0.54±0.05, P=0.041), whereas no differences were found in tongue and temporalis (*Fig. 3B*).

DISCUSSION

Mdx skeletal muscles of torso, diaphragm and limbs often present varying patterns of histopathology (28). In a recent

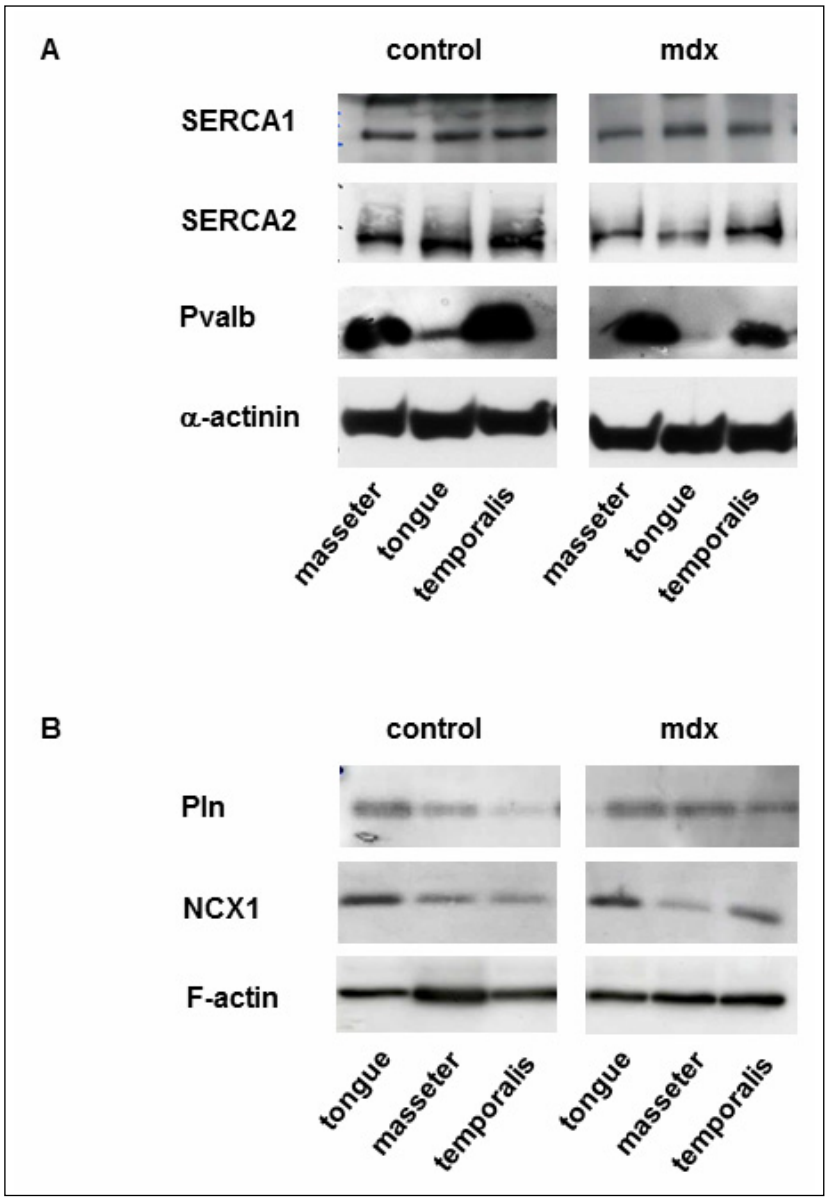

Fig. 1. Western blot analyses in masticatory muscles of control and mdx mice. (*A*): Representative Western blots of SERCA1, SERCA2 and parvalbumin in masseter, temporal muscle as well as tongue from control and mdx mice. A monoclonal antibody was used to detect α-actinin serving as an internal control. (*B*): Representative Western blots of NCX11 and phospholamban in masseter, temporal muscle as well as tongue. A monoclonal antibody was used to detect F-actin serving as an internal control.

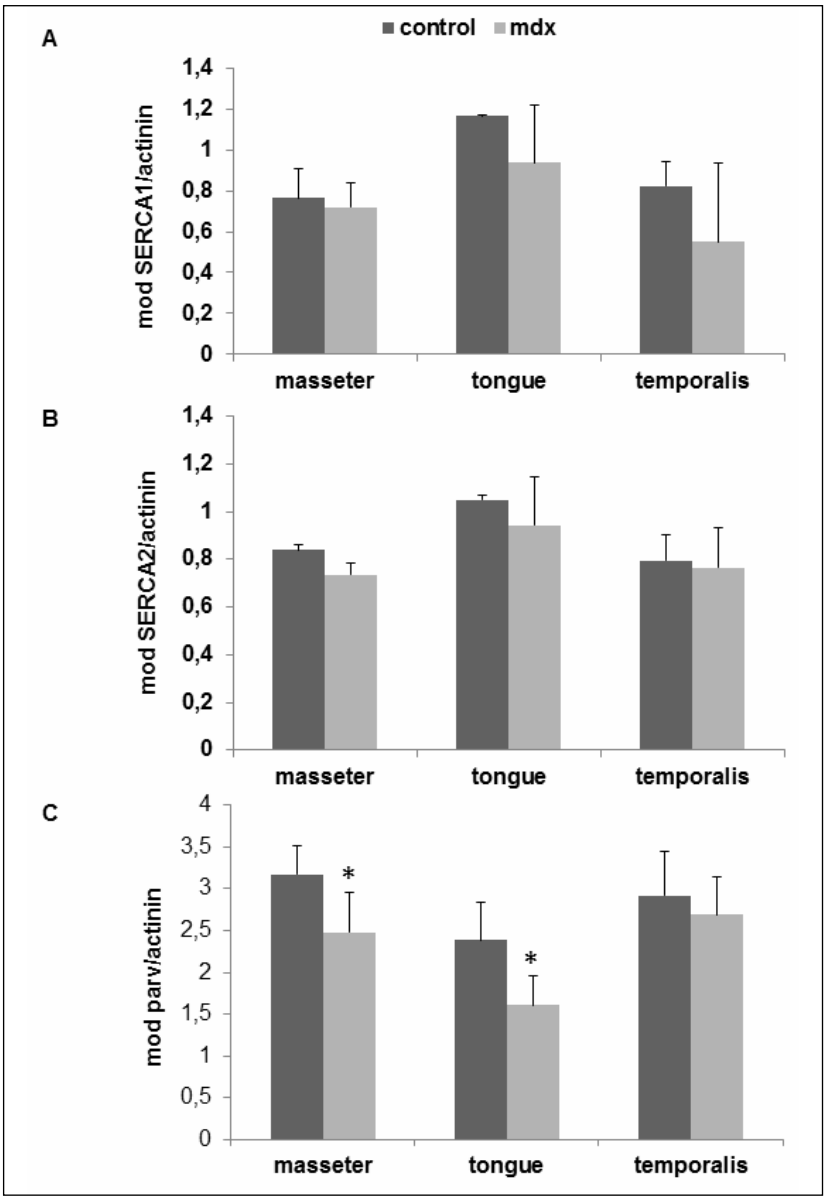

Fig. 2. Quantitative analyses of (*A*) SERCA1, (*B*) SERCA2 and (*C*) parvalbumin western blots as shown in *Fig. 1*. Protein bands attributed to SERCA1, SERCA2 and parvalbumin as well as α-actininwere evaluated using the GelScan 5.2 software (Serva, Germany). The mean optical densities (mod) ±S.D. of control and mdx mice are given in all cases for 3 independent experiments. *P<0.05; significant differences between control and mdx mice; paired t-test.

study from our laboratory we confirmed such inter-muscular differences in masticatory muscles of 100-days-old mdx mice (23). This work is a follow up study of the above mentioned studies on mdx orofacial muscles because we wanted to know why the orofacial muscles were affected differentially by this disease. Muscle activity and function appear to be related to ionic concentrations in the muscle (10). It is well known that aberrances in the intracellular Ca^{2+} concentration play an important role in the excitation-contraction-relaxation processes in muscular tissue. Defects in the Ca^{2+} regulating proteins result in abnormal chronic and acute Ca^{2+} concentrations, which can lead to various forms of impaired muscle function (16). On the other hand, enhanced Ca^{2+} buffering has been proposed as a contributory mechanism to explain escape damage in the presence of excess intracellular calcium. Previously, it was shown that the dystrophic phenotype in δ-sarcoglycan-null mice and mdx mice is dramatically improved by skeletal muscle-specific overexpression of SERCA1 (29). SERCA1 overexpression reversed a defect in sarcoplasmic reticulum Ca^{2+} reuptake that characterizes dystrophic diseases.

In this study, we found higher gene and protein expression of the Na^+/Ca^{2+} exchanger NCX1 in the dystrophic masseteric muscle. NCX1 is known to have a high capacity for calcium transport (30). Recently, Deval and coworkers (30) could demonstrate that the withdrawal of external sodium ions generate elevated intracellular calcium activity in isolated myotubes. Furthermore, it was shown that the amplitudes of the Na^+-free induced $[Ca^{2+}]_{int}$ rises were generally higher in myotubes from DMD patients compared to controls. Imbert *et al*. (31) found an abnormal elevation of the resting calcium level in DMD myotubes. An upper activity of the exchange mechanism due to an elevated $[Ca^{2+}]_{int}$ in DMD myotubes can be suggested. It is known that NCX1 is regulated by cytoplasmic calcium (32, 33). Mackiewicz and Lewartowski (34) have found that the effect of SR Ca^{2+} leak from ryanodine receptors is strictly dependent on the activity of NCX1 and SERCA.

Cully and coworkers (35) demonstrated that PMCAs are up-regulated in mdx muscles. Using western blot analyses a 2fold increase in the expression of PMCAs in dystrophic Extensor digitorum longus (EDL) muscles were detected compared to controls. In our study, increased expression levels for PMCA4

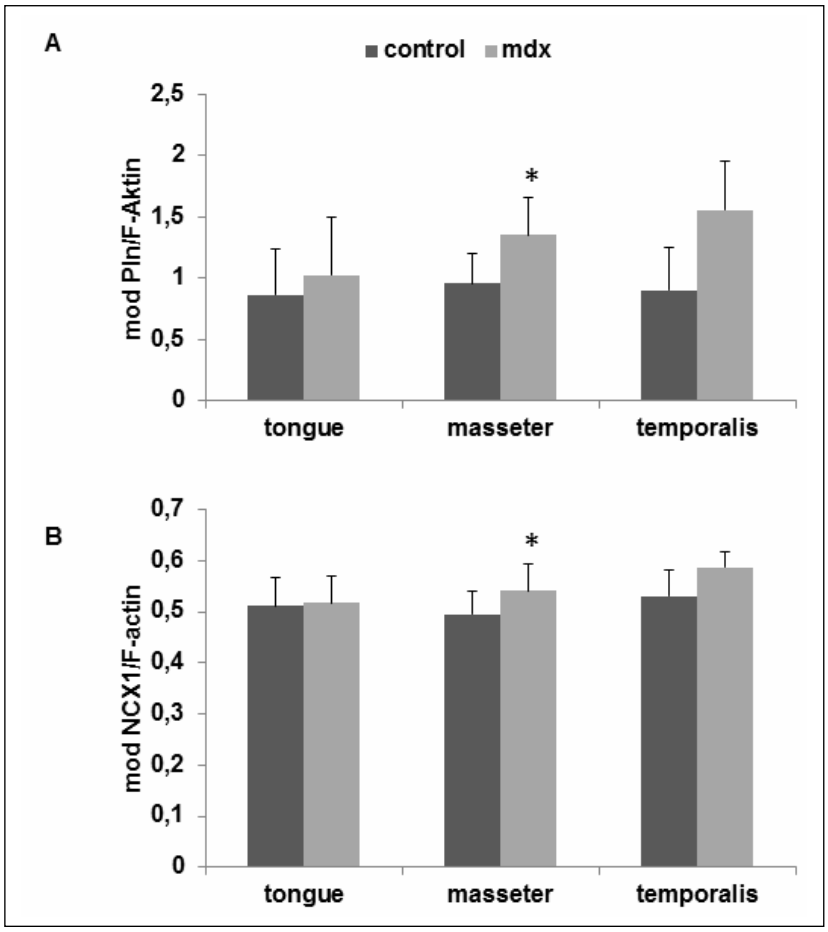

Fig. 3. Quantitative analyses of (*A*) phospholamban and (*B*) NCX1 western blots as shown in *Fig. 1*. Protein bands attributed to phospholamban and NCX1as well as F-actin were evaluated using the GelScan 5.2 software (Serva, Germany). The mean optical densities (mod) ±S.D. of control and mdx mice are given in all cases for 3 independent experiments. *P<0.05; significant differences between control and mdx mice; paired t-test.

were also found in dystrophic temporalis muscular tissue, whereas the amount of PMCA1 mRNA was significantly reduced in tongue muscles of mdx mice. The plasma membrane Ca^{2+}-ATPase is known to be activated due to the release of sarcoplasmic reticulum Ca^{2+}. Interestingly, a reduction in SERCA activity has been observed in dystrophic muscles (36-38). This is in contrast to our findings in orofacial muscles. We did not find any changes in the expression of SERCA1 and SERCA2 in masseteric and temporal muscles as well as in tongue muscular tissue.

An up-regulated mRNA expression of phospholamban, sarcolipin, parvalbumin and Cacna1s was found in extraocular muscles (EOMs) myotubes compared to tibialis muscle myotubes (20). In fully differentiated adult tissue from normal and dystrophic mice the following genes showed significantly higher expression in EOMs compared to tibialis muscle: SERCA2, PMCA1, PMCA4, NCX1, phospholamban and sarcolipin (20, 39). Furthermore, the expression levels of these proteins in EOMs did not differ between control and mdx mice (39). EOMs are clinically and histologically spared in DMD patients and mdx mice and these muscles are more resistant to elevated Ca^{2+} levels than limb muscles (19).

From all these results obtained in this study compared with those from the literature it can be concluded that masseteric and temporal muscular tissue of 100 day old mdx mice might be more resistant to elevated Ca^{2+} levels than tongue muscles. Recently, it was shown that mdx masseter and temporal muscles consist of mostly regenerated fibers with central nuclei and show just few inflammatory foci and increased collagen content. The degree of centronucleation, the mean fiber diameter as well as the amount of collagen did not significantly differ between masseter, temporal and soleus muscle of mdx mice (23). In further studies could be also shown that masticatory muscles has many similarities in the expression profile to the soleus muscle (21, 24, 40, 41). Furthermore, the studies on the expression of the calcium-regulating genes showed no significant differences between the masseter, temporalis and soleus (unpublished data). In contrast, dystrophic tongue muscles contain only 11.2% of regenerated muscle fibers and inflammatory foci are hardly detectable (23). At this histological finding is to realize that the muscle fibers of mdx masseter and temporalis have already undergone a cycle of muscle fiber degeneration followed by regeneration. Furthermore, it was speculated that mdx tongue muscle seemed to be almost hit by this disease. It is well known that in 2–9 weeks old mdx mice muscle fibre degeneration is dominating (42). From the fifth week of life, the muscle fiber regeneration prevailed in the mdx mice, so that there was no further muscle wasting and the animals did not show any signs of muscle weakness up to the age of two years (43, 44). Therefore our findings suggest that regenerated fibres in 100 days old mdx masticatory muscles express Ca^{2+} handling protein characteristic for EOM muscles.

In conclusion, our findings revealed that mdx masticatory muscles showed an unequally altered expression of genes involved in the Ca^{2+} homeostasis that can support the differences in masticatory muscles response to dystrophin deficiency. Regenerated dystrophic fibres of masticatory muscles expressed Ca^{2+} handling protein characteristic for EOM muscles and seemed to be protected against calcium overload. In contrast, dystrophic tongue seems a priori secured from muscle

degeneration even though in this muscle we found expression levels of Ca^{2+} handling proteins typical for dystrophic muscles. These characteristics provide insight for the potential role of superior calcium buffering in dystrophic mice muscles and its possibility of muscle regeneration. Further studies should find out, if the expression of genes and proteins involved in Ca^{2+} handling is age-dependent and wether the expression is associated with muscle degeneration/regeneration.

Conflict of interests: None declared.

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