

Expression rate of myogenic regulatory factors and muscle growth factor after botulinum toxin A injection in the right masseter muscle of dystrophin deficient (mdx) mice

Ute U. Botzenhart^{1,C–F}, Ricarda Gerlach^{1,B}, Tomasz Gredes^{1,C–F}, Ines Rentzsch^{2,B}, Tomasz Gedrange^{1,F}, Christiane Kunert-Keil^{1,A–F}

¹ Department of Orthodontics, Carl Gustav Carus Campus, Technische Universität Dresden, Germany

² Department of Anesthesiology and Intensive Care Medicine, University Hospital Carl Gustav Carus, Technische Universität Dresden, Germany

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Address for correspondence

Ute Botzenhart

E-mail: Ute.Botzenhart@uniklinikum-dresden.de

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Abstract

Background. The mdx mouse, the most approved animal model for basic research in Duchenne muscular dystrophy (DMD), has the ability to compensate muscle degeneration by regeneration process, which is obvious at approx. 3 months of age. Hence, this mouse model is only temporarily suitable to proof craniofacial changes which are usually evident in humans with the progression of the disease.

Objectives. The purpose of our study was to examine the impact of botulinum toxin A (BTX-A) in influencing muscle regeneration in the masticatory muscles of healthy and mdx mice.

Material and methods. Chemo-denervation of the right masseter muscle was induced in 100-day-old, healthy and dystrophic mice by a specific intramuscular BTX-A injection. Gene expression and protein content of myogenic regulatory factors and muscle growth factor (MyoD1, myogenin and myostatin) in the right and left masseter, temporal and the tongue muscle were determined 4 and 21 days after injection, respectively, using quantitative reverse transcription polymerase chain reaction (qRT-PCR) and western blot technique.

Results. The 4 day and 21 day interval proved significant but varying changes of mRNA expression in both control and mdx mice. At the protein level, myogenin expression was increased in the temporal and masseter muscle on the injection side in controls, whereas dystrophic mice showed the same effect for MyoD1 expression. Additionally, increased protein expression of all studied genes could be found in dystrophic mice compared to controls, except the left temporal and the tongue muscle.

Conclusions. Muscle regeneration is not constant in BTX-A injected mdx masticatory muscles, presumably due to the already exhausted capacity or functional loss of satellite cells caused by dystrophin deficiency, and, therefore, disturbed regeneration potential of myofibrils. Botulinum toxin A injection cannot fully break down regulatory processes at molecular level in 100-day-old mdx mice. Further investigations are necessary to fully understand the regeneration process following BTX-A injection into dystrophic muscles.

Key words: myostatin, BTX-A, MyoD1, myogenin, mdx mice

Introduction

Skeletal muscles, the most abundant tissue of the body, are composed of myofibers and grow in size by fusing postnatal muscle stem cells, called satellite cells. These mononucleated cells are located between the plasma membrane and the basal lamina that surrounds each muscle fiber. They play a key role in the regeneration of adult skeletal muscles by compensating for daily muscle stress and strain and can be activated after muscle injury.¹ Satellite cells are affected by several growth factors as well as transcription factors, such as members of the myogenic regulatory factor (MRF) family. Among them, myogenic transcriptional regulators, such as MyoD and myogenin, are essential for the proliferation of satellite cells and for the development of early regenerating fibers, even in dystrophic muscles. MyoD is considered as one of the key regulatory factor of muscle regeneration.² Based on numerous studies, it could be demonstrated that MyoD is required for the determination of skeletal myoblasts, whereas myogenin plays a decisive role in the expression of the terminal muscle phenotype, regulating skeletal muscle metabolism and exercise capacity during adult life.³ Increased myopathy was observed by a lack of MyoD, which had a negative effect on the embryonic formation, postnatal survival and function of satellite cells, and thus an adverse influence on muscle formation. In contrast, the growth factor myostatin has a negative impact on the postnatal muscle growth, due to its suppressive effect on satellite cell activation, proliferation and self-renewal, as well as myoblast proliferation and differentiation.^{4,5} The proliferation and differentiation of satellite cells during muscle repair is largely influenced by vascularization, innervation, hormones, nutrition, and the extent of muscle damage, which takes place either under physiological or pathological conditions, for example in muscular dystrophies.⁶

Muscular dystrophy is a general term that covers a diverse group of inherited myogenic disorders characterized by progressive muscle wasting and degeneration among them Duchenne muscular dystrophy (DMD) is the most common one. It results from mutations in the dystrophin gene and lack of its functional protein. The absence or enormous reduction of functional dystrophin protein results in muscle fiber necrosis without further regenerative processes. At the beginning of the disease, new muscle fibers are formed by satellite cells or by fusion of resistant myoblasts. In more advanced stages of DMD, inadequate muscle regeneration, probably due to loss of satellite cells, which after many rounds of muscle degeneration and regeneration processes become “exhausted”, leads to the degeneration of skeletal muscles, which are then replaced by fatty and connective tissue.⁷ Additionally, loss of muscle tissue is not homogenous, but involves specific muscle groups.¹ Proximal muscles of the extremities are affected first, followed by upper arms and upper legs. Interestingly, approx. 2 years after the patient had become wheelchair-bound, the orofacial muscles are affected, which results

in muscle imbalance and severe craniofacial and dental abnormalities.⁸ This affirms the assumption that a relationship between muscle dysfunction and craniofacial morphology may exist.

In contrast to DMD patients, skeletal muscles of the mdx mouse, the most commonly used animal model for DMD, can regenerate throughout life. Ongoing muscle degeneration can be found in mdx mouse muscles, which usually peaks at 3 weeks of age, and is characterized by a decrease of myofibers and pathological features becoming more severe with age.⁹ Due to a spontaneous and effective recovery of muscle cells, compared to humans, the mdx mouse has a modest dystrophic phenotype, exhibits a more benign progression of the dystrophy, and can, therefore, not accurately be compared with human DMD disease pattern.¹⁰ Nevertheless, it will be assumed that the myogenic activity of satellite cells in mdx muscles also appears to be depleted with age.¹¹

Botulinum toxin is a purified form of the neurotoxin derived from the bacterium *Clostridium botulinum* responsible for botulism. Following local injection into the muscle, the toxin inhibits the vesicular release of acetylcholine (ACh) neurotransmitter at the neuromuscular junction producing chemical denervation and paralysis of the striated muscles in humans and animals.¹² Resulting muscle paralysis and safety of botulinum toxins have permitted their widespread use in a variety of therapeutic applications, such as unloading the jaws, alleviation of facial pain involving the temporomandibular joint, masticatory myalgia, sialorrhoea, bruxism, and hemifacial spasm.¹³ Hence, with botulinum toxin A (BTX-A) injection just a temporary paralysis is possible, because in healthy muscle tissue after a certain time, reinnervation occurs. At the neuromuscular junctions of humans, functional recovery of the nerve terminal takes 2–4 months, whereas in rats and mice functional muscle recovery is reported to be much faster.¹⁴ The onset of toxin action in rodents is 24 h after injection, usually peaks at 2 weeks and paralysis lasts 4–6 weeks.¹⁵

Research in the field of orthodontics as well as in yet unpublished data of our own research group in the mdx mouse during maximal dystrophic muscle degeneration could already show a relationship between muscle weakness and craniofacial deformities, which had originally been described by Melvin L. Moss as the so called “functional matrix theory”.⁸ Due to recovery, in the mdx mouse these effects disappear, so that this mouse model cannot adequately be used to scientifically prove this correlation. Thus, to analyze muscle-function influence on craniofacial bone growth and development in a sufficient way, a specific and durable muscle dysfunction should be induced. As it is known that BTX-A causes muscle paralysis as well as muscle degeneration in healthy muscle tissue, this toxin might also be able to trigger dystrophic features in mdx mice, making this mouse model comparable to the human disease of DMD and accessible for research in the craniofacial region. Hence, we wanted to examine whether BTX-A is suited to generate a sustained dystrophy in the mdx mouse masticatory

muscles by verifying these effects on the basis of regeneration processes. In a recently published paper about MyHC expression in the masticatory muscles of 100-day-old mice following the same protocol, a single specific intramuscular BTX-A injection in the right masseter muscle induced changes of MyHC expression in healthy mice, indicating a shift to type I fibers and simulating dystrophic features in these mice, whereas dystrophic muscles did not react to BTX-A injection.¹⁶ In contrast to the abovementioned results, on the one hand our research group could find changes in caveolin 1, caveolin 3 and VEGF protein expression in dystrophic mice after a single injection of that toxin in the right masseter muscle with raised expression of all studied proteins, whereas in the right masseter muscle of controls a decrease of caveolin 3 expression, due to BTX-A injection, could be found. On the other hand, mRNA expression was unchanged in both mouse strains.¹⁷

The aim of the present study was to examine BTX-A influence on the regenerative capacity in mdx masticatory muscles by identifying MRFs and a muscle growth factor as markers for muscle regeneration and repair after a single specific intramuscular BTX-A injection in the right masseter muscle of healthy and dystrophic mice, and to evaluate if this drug was able to induce or prologue dystrophic features in the masticatory muscles of these mouse strains.

Material and methods

Animals and experimental protocol

Male and female mice of the inbred strain C57BL-10ScSn (control group, n = 20) and C57BL/-Dmdy (mdx) (test group, n = 25), originally obtained from Jackson Laboratory (Bar Harbor, USA) and borne in the Laboratory Animal Experimental Bioassay Centre Dresden (experimental centre of the medical faculty, TU Dresden, Germany), were used in this study. At the beginning of the experiments, mice of both strains were aged 100 days and had a body mass of approx. 30 g. All procedures performed in this study were approved by the Laboratory Animal Research Committee of Saxony (Germany) with the No.: 24–9168.11–1/2013–46. An intraperitoneal injection consisting of a mixture of 10% ketamine (Ceva Tiergesundheits GmbH, Düsseldorf, Germany) and 2% Rompun® (Bayer, HealthCare AG, Leverkusen, Germany) at a ratio of 3:2 (0.1 mL per 100 g body mass) was used for temporal anesthesia. Chemodenervation was induced by a single specific intramuscular injection of 0.025 mL BTX-A (Botox®; Allergan, Irvine, USA; 1.25 IU/0.1 mL in physiologic NaCl solution) in the superficial and deep venter of the right masseter muscle as described recently by Botzenhart et al.^{16,17} After injection both healthy and mdx mice were randomized into 2 groups according to the postinjection periods of 4 days (T1; control group: n = 7; test group: n = 9) and 21 days (T2; control group: n = 13; test group: n = 16), respectively.

Due to the fact that paralysis of masseter muscle is usually evident 3 days after injection by teeth chattering and the refusal of solid food, during the first 7 postinjection days soft food was offered additionally.¹⁶ After 4 days (T1) and 21 days (T2), the mice were painlessly killed using an overdose of isoflurane. Immediately, the head was separated from the body and samples of the following muscles were carefully dissected by the same trained observer: right and left masseter muscle, right and left temporal muscle and tongue muscle. Samples were immediately frozen in liquid nitrogen (–173°C) and stored at –80°C until further processing. Muscles harvested, corresponded to the superficial and (in parts) the deep masseter muscle, the medial temporal muscle and the flexible part of the tongue, including the internal tongue muscles.¹⁸ For both investigation periods, mRNA expression and protein content of MRFs, MyoD1 and myogenin, and muscle growth factor, myostatin, were analyzed. Some of the samples examined in this study had already been used for quantification of MyHC isoforms as well as caveolin 1, caveolin 3 and VEGF expression.^{16,17}

Quantitative reverse transcription polymerase chain reaction

The isolation of total RNA and its reverse transcription in cDNA was carried out exactly as described by Botzenhart et al.^{16,17}

Gene expression analysis of the myogenic differentiation factors *MyoD1* and myogenin (*Myf4*) as well as muscle growth factor myostatin (*MSTN/GDF8*) in the extracted muscle samples was performed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) using specific TaqMan PCR probes and primers (Taq-Man® Assays: *MyoD1*: Mm00440387_m1; *Myf4*: Mm00446194_m1; *MSTN*: Mm03024050_m1; PE Applied Biosystems, Weiterstadt, Germany) and the TOptical cycler (Analytik Jena AG, Jena, Germany) as described previously.^{16,17}

The 2-ΔΔCt method was used in order to quantify the studied genes in mdx mice relative to controls in relation to those of 18s rRNA (Eukaryotic 18S rRNA Endogenous control: 4310893E; PE Applied Biosystems).¹⁹

Western blot

Muscle protein which had been isolated from each murine tissue sample, following the protocol described earlier, was loaded onto Criterion™ TGX Stain-free™ Precast Gels (Bio-RAD Laboratories GmbH, Munich, Germany) for 60 min under constant voltage of 100 V, and after separation, transferred to PVDF membranes (Trans-Blot® Semi-Dry transfer system, Trans-Blot® Turbo™ Midi PVDF Transfer Packs; Trans-Blot® Turbo™ blotting apparatus; Bio-RAD Laboratories GmbH).¹⁶ Dry milk (5%) in phosphate-buffered saline (PBS) buffer with 0.05% tween at 4°C was used overnight to block the western blot membranes.

Specific antibodies against myogenin (EPR4789; Abcam, Cambridge, UK), MyoD1 (C20; Santa Cruz, Heidelberg, Germany) and myostatin (AB 71808; Abcam) were used for incubation, followed by horseradish peroxidase (HRP)-conjugate goat anti-mouse or anti-rabbit immunoglobulins (1:5,000; Dako, Hamburg, Germany).

Bound antibodies were detected and visualized with an enhanced chemiluminescence system (WesternBright Chemiluminescence Substrate Quantum; Advantia Inc., Menlo Park, USA). In order to calculate the protein content on each gel, monoclonal anti-glyceraldehyde-phosphate dehydrogenase (GAPDH) antibody (clone 6C5; 1:1000; Millipore, Billerica, USA; incubation for 2 h at room temperature) served as loading control. GelScan 5.2 software (Serva, Heidelberg, Germany) was used to quantify protein bands (mean optical density $\pm$ standard error of the mean [SEM]), in each case of $n = 3$ different muscle samples (different animals) and 2 independent western blot analysis.

Statistical analysis

Statistical analysis for evaluation of differences in mRNA expression and protein content of the investigated MRFs and muscle growth factor in the extracted muscle samples after BTX-A treatment was performed using SigmaStat v. 3.5 (Systat Software Inc., San Jose, USA). In the case of normal distribution unpaired t-test and otherwise, the Mann-Whitney U test was used (significance level: $p \leq 0.05$).

Results

As no gender differences could be observed, in all examinations no distinction between male and female mice was made.

mRNA expression 4 days after BTX-A injection

When considering the gene expression of *Myf4*, no differences in mRNA amount were found between BTX-A-treated and untreated masseter muscle for both, control and mdx mice. In contrast, compared to the left side, in the right temporal muscle a 4.6-fold as well as a 2-fold increase in *Myf4* gene expression could be detected in control and mdx mice, respectively (Fig. 1A).

Four days after BTX-A injection in the right masseter muscle of control mice the mRNA expression of *MyoD1* was significantly increased in the treated masseter muscle compared to the untreated muscle tissue ($p = 0.021$), whereas no significant differences in mRNA amount were detected in dystrophic mice. At the same time, a 3.6-fold increase in *MyoD1* mRNA expression could be observed in the right temporal muscle of controls compared to the left side (Fig. 1B).

For *MSTN* decreased levels of the mRNA expression could be detected in the right masseter muscle of dystrophic mice, whereas the expression counted only 35.8% of that found in the left untreated masseter muscle.

In contrast, in healthy mice no differences in mRNA amount of *MSTN* were found between treated and untreated masseter muscle. In those animals, however, significant decreased *MSTN* expression by 50% was detected in the right temporal muscle compared to the left side, which was not found in mdx mice (Fig. 1C).

Furthermore, significant differences were detected between healthy and dystrophic mice, e.g., in the left masseter muscle for *Myf4* and *MSTN*, and in the right temporal muscle for *MyoD1* and *MSTN* (Fig. 1).

mRNA expression 21 days after BTX-A injection

Twenty-one days after BTX-A injection, significant differences could be detected in both healthy and dystrophic mice, including a 6.2- and 6.0-fold increase in mRNA

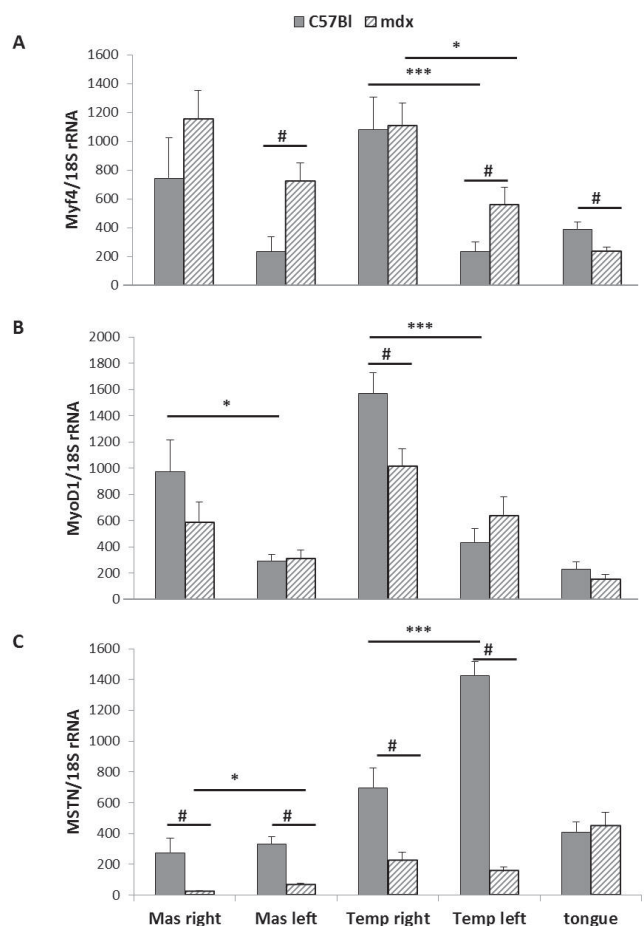


Fig. 1. Myostatin, myogenin and MyoD1-mRNA expression in treated and untreated muscle tissue 4 days post-toxin injection in the right masseter muscle of healthy and mdx mice

Means $\pm$ standard deviations (SD) for controls ($n = 7$) and mdx mice ($n = 9$); Mann-Whitney U test; * $p \leq 0.05$ treated vs untreated masseter muscle; # $p \leq 0.05$ control vs mdx; * right vs left.

expression for *Myf4* in the BTX-A-treated masseter muscle compared to the left side, respectively (Fig. 2A) and a 3.7-fold increase in *MyoD1* mRNA amount in the BTX-A-treated masseter muscle of control and mdx mice, respectively (Fig. 2B).

Only 22% as well as 53% of the *MSTN* mRNA amount of the left side could be found in the right masseter and right temporal muscle of control mice (Fig. 2C), and reduced *MSTN* mRNA levels in dystrophic mice could be detected compared to healthy animals in the left masseter and left temporal muscle (Fig. 2C).

Protein content 21 days post-injection

Quantitative analysis of specific protein bands for myogenin, MyoD1 and myostatin, respectively (Fig. 3), revealed the following results: a 1.7-fold and 1.9-fold increase of myogenin expression on the right side compared to the contralateral side for both, masseter and temporal muscle in control mice (Fig. 4A). In dystrophic mice, significantly

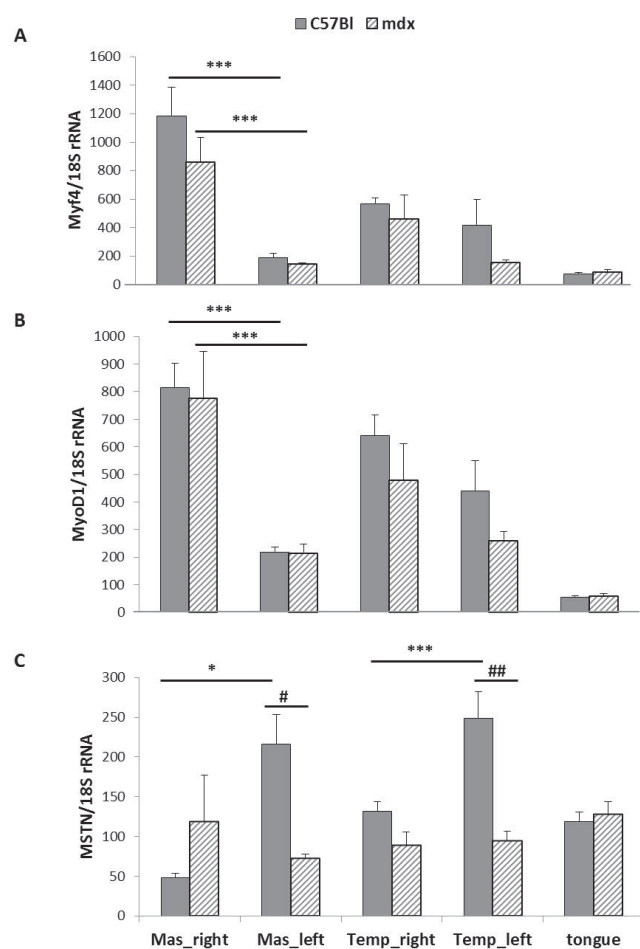


Fig. 2. Myostatin, myogenin and MyoD1-mRNA expression in treated and untreated muscle tissue 21 days post-toxin injection in the right masseter muscle of healthy and mdx mice

Means $\pm$ standard deviations (SD) for controls ($n = 10$) and mdx mice ($n = 13$); Mann-Whitney U test; * $p \leq 0.05$ treated versus untreated masseter muscle; # $p \leq 0.05$ control vs mdx; * right vs left.

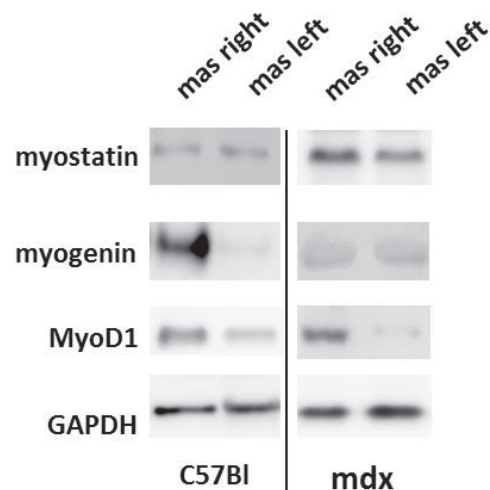


Fig. 3. Detection of myostatin, myogenin and MyoD1 in masseter muscle. Representative western blots of the right (injected) and left (non-injected) masseter muscle of control (C57Bl) and mdx mice. A monoclonal antibody was used to detect glyceraldehyde 3-phosphate dehydrogenase (GAPDH) serving as an internal control

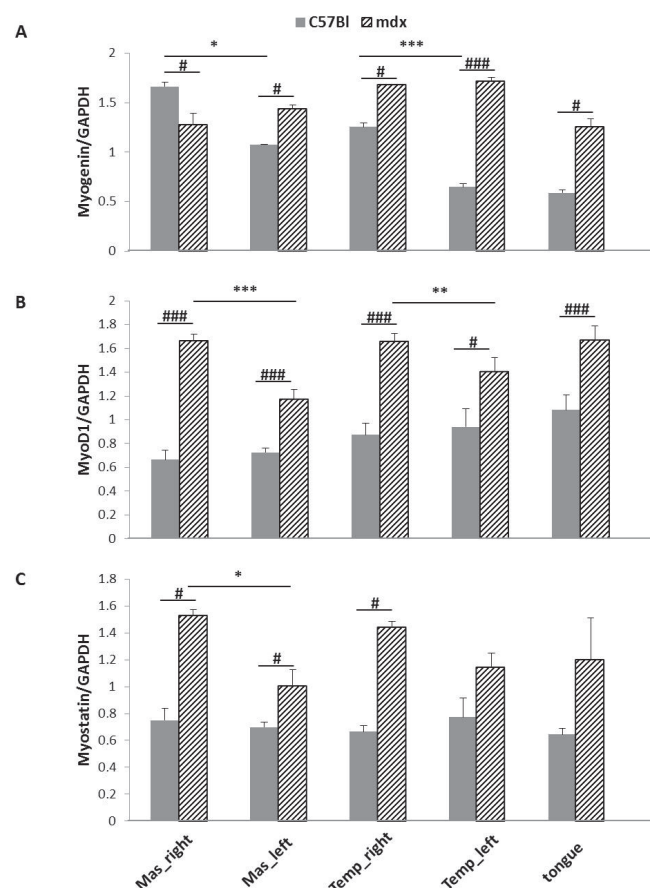


Fig. 4. Quantitative analysis of myostatin, myogenin and MyoD1 western blots in masseter, temporal and tongue muscle of mdx and control mice 21 days after BTX-A injection in the right masseter muscle of healthy and mdx mice

Protein bands attributed to myostatin, myogenin and MyoD1 were evaluated using GelScan 5.2 software (Serva, Heidelberg, Germany); mean optical densities (MOD) $\pm$ standard error of the mean (SEM) of control and mdx mice are given in all cases for $n = 3$ muscle samples (different animals) and 2 independent experiments (* $p \leq 0.05$ right vs left; # $p \leq 0.05$ control vs mdx).

increased protein levels of MyoD1 as well as myostatin were found in the BTX-A-treated masseter compared to the untreated muscle. The same changes were also found in temporal muscle in the case of MyoD1 (Fig. 4).

Furthermore, significantly increased protein amounts for myogenin, MyoD1 and myostatin could be detected in almost all dystrophic muscle samples compared to controls, with the exception of the left temporal and the tongue muscle in case of myostatin (Fig. 4).

Discussion

In this study 2 postinjection time points were analyzed to examine the time course of muscle recovery and regeneration in BTX-A-injected masseter and noninjected masticatory muscles; a 4-day interval reflecting the full characteristics of toxin effect and a 21-day interval illustrating changes after full paralysis and induction of myogenesis. The expression analysis of the genes and proteins, which are known to play a key role in skeletal muscle development and regeneration, demonstrated that the repair of healthy and dystrophic muscles occurred at staggered intervals. Furthermore, the gene analysis of *Myf4*, *MyoD1* and *MSTN* after 4 and 21 days allowed for better insight into the molecular mechanisms involved in muscle regeneration after muscle paralysis induced by BTX-A. Earlier studies have shown that MyoD protein expression in diaphragm, quadriceps and intrinsic laryngeal muscles do not show any significant differences between control and mdx mice at the age of 1, 4 and 9 months.²⁰ The same could be demonstrated for the expression of myogenin and MyoD1 protein in the masticatory muscles of 100-day-old dystrophic mice compared to the controls.²¹ For this reason, the present study was performed using 100-day-old control and mdx mice. On the 4th day after BTX-A injection, by means of gene expression in the right masseter muscle, it could be concluded that the full effect of muscle paralysis was obtained. A significantly increased expression of *MyoD1* suggests that regenerative processes in the right masseter muscle of controls had already begun. This is in accordance with previous findings of Hatade et al.²² Recently, it has been shown that 3 days after BTX-A injection, full paresis of the muscle was present and electrical stimulation failed to elicit any visible muscle contraction.²³ Between 3–7 days, initial sprouting occurred and at 4 days remodeling of these poisoned terminals became apparent. SNAP-25, a synaptic membrane protein that is involved in the vesicle exocytosis process, was present 9 days post-injection; however, only a minor reorganization of Ach receptors could be found.²³ In our study, gene analysis after 4 days revealed that healthy mice responded faster to muscle paralysis indicated by initiation of muscle regenerative and adaptive processes, while in mdx mice these processes could not be determined at that time. Furthermore, due to the enhanced expression of *MyoD1*

and *Myf4* and the decreased gene expression of *myostatin* in healthy mice, it can be assumed that the right temporal muscle supported masticatory function after the paralysis of the right masseter.

Three weeks after BTX-A injection, an increase of both *MyoD1* and *Myf4* genes could be detected in the right masseter muscle of healthy and mdx mice. At a molecular level, control animals as well as dystrophic mice showed a similar development of regenerative processes. However, BTX-A-induced paralysis of the right masseter muscle was diminished after 3 weeks, indicating that a reinnervation of the treated muscle had already begun by sprouting new nerve branches. It has previously been reported that the injection of animal neurotoxins in mice muscles accelerated the recovery of neurotransmission, and BTX-A treatment is known to result in rapid initiation of neurite sprouting from the paralyzed nerves.²³ The process of axon sprouting plays a key role in the subsequent recovery of paralyzed motor endplates and is a striking example of synaptic plasticity, which allows survival and, eventually, complete recovery from BTX-A-induced neuroparalysis.²³ Recovery from BTX-A-induced paralysis can be divided into different sections, at the neuromuscular junction (NMJ) as well as in the muscle fibres, which do not run synchronically. Thus, functional recovery of muscles is not concomitant with recovery of the NMJ. Furthermore, it should be mentioned that short-term denervation stimulates satellite cells to proliferate, whereas long-term denervation exhausts the satellite cell pool.²⁴ It is known that nerve terminal regeneration and functional reinnervation after BTX-A injection saw progress within 28–30 days and the first functional resumption of muscle activity appeared.²⁵ Usually, between 3–7 days, initial sprouting occurs and remodeling of these poisoned terminals becomes apparent at 4 days.²³ Furthermore, recovery of the NMJ was accompanied by an upregulation of Ach receptor subunits. Hence, after BTX-A injection most Ach subunit mRNAs are upregulated early and return to normal levels by approx. 2 weeks, so that 18–28 days after intoxication reorganization and clustering of Ach receptors facilitates reinnervation, and by 28 days, the first muscle twitch is possible.²⁶ Thus, it is apparent that neurotransmission is mediated by functional synapses formed between the terminal sprouts and the muscle fibers and these branches can at least temporarily adopt the function of the parent terminals in muscles paralyzed by BTX-A. Within 42–63 days after BTX-A-induced paralysis, sprouts receded, and the original nerve terminals immediately returned to functionality, and up to 3 months endplates regained morphologies and patterns indistinguishable from those visualized before poisoning.²³ *MRF4* and *myogenin* are significantly elevated compared to control levels at 3 days following BTX-A injection and return to control levels by 30 to 90 days after injection, while mRNA levels of *Myf5* and *MyoD* do not significantly change.²⁷ Recently, it has been demonstrated that BTX-A treatment significantly increased MyoD

expression in functionally denervated extraocular muscles, while a population of activated satellite cells became stably integrated into existing myofibers.²⁸ It is well-known that MyoD and myogenin are expressed in activated mononuclear muscle precursor cells and that they are the nodal point during the specification of the myogenetic lineage. The enhanced expression of these genes is associated with the growth of skeletal muscles in embryonic development, followed by a neonatal decrease. MyoD knock-out mice have no macroscopic degenerative phenotype under normal conditions but show significant deficiencies in regeneration.²⁹ It was postulated that, in the absence of MyoD, some essential steps in the myogenic progression were blocked, leading to a population of activated satellite cells, which returns to a quiescent stage. These satellite cells also exhibited major differences in myogenic gene expression, e.g., failure in up-regulation of *MRF4* and *myogenin*.²⁹ However, in the mdx mouse, elevated *MyoD* and *myogenin* expression was detected at about 21 days of age, at the postnatal onset, at which regenerative activity is first observed in dystrophic muscles.³⁰ The postnatal levels of these genes were not reduced to those observed in the control mice and these maintained expression of *MyoD* and *myogenin* confirmed the regenerative processes in the skeletal muscles of adult mdx mice.³⁰ However, it has been proven that the deletion of *myogenin* in mdx mice neither reversed the pathologic effects of dystrophy nor aggravated their DMD, indicating that myogenin is dispensable for muscle regeneration in adult mice.³¹ The expression of both, *MyoD1* and *myogenin* genes, is necessary in the regenerative process, for the proliferation of myoblasts and for the development of early regenerating myotubes, even in dystrophic muscles. Within the active regenerating process these 2 genes were expressed at reasonably high levels in mdx mice after a single administration of bupivacaine hydrochloride, which is in accordance with our study. In the case of BTX-A administration, in comparison to untreated mice, in mdx mice a strongly increased MyoD expression could be detected at protein level.

The loss of myogenin during adult life confers a resistance to denervation-induced muscle atrophy.³² After BTX-A injection myogenin upregulation usually persists for a longer period, which is also in accordance with our results. At protein level the right masseter muscle of controls showed a significant increase in myogenin expression. Entering myogenic differentiation a dysfunction of satellite cells has recently been discussed in dystrophic muscles due to the loss of polarity, abnormal division patterns including centrosome amplifications, impaired mitotic spindle orientation, and prolonged cell divisions based on the lack of dystrophin in mdx muscle stem cells.³³ This may explain the results found at protein level with a lack of myogenin expression in BTX-A-treated masseter muscle compared to healthy controls, whereas MyoD1 expression in the right masseter muscle of mdx mice was upregulated, indicating a delay in differentiation.

It has been proven that myostatin activity determines skeletal muscle mass. The inhibition of *myostatin* gene promotes muscle regeneration in humans and in animals in the postnatal period by increasing muscle mass and strength, and it has been shown that the inhibition of *myostatin* could be effective for increasing muscle mass and preventing muscle degeneration even in adults.³⁴ In contrast, skeletal muscle atrophy is associated with increased expression of this growth and differentiation factor. The significantly reduced gene expression of *MSTN* in the right masseter and temporal muscle of control animals might therefore be explained by an advanced regenerative process of the paralyzed muscle and/or a continuing compensatory effect of its neighboring muscle. However, in dystrophic muscles no significant changes regarding *MSTN* expression could be found at that time point. Several studies could demonstrate a significant effect of myostatin postnatally in muscle misuse and wasting.³⁵ Mice lacking myostatin induced a widespread increased muscle mass resulting from both hyperplasia and hypertrophy.³⁶ In mdx mice in which *myostatin* was knocked out or postnatally inhibited a less severe phenotype with greater absolute force and less fibrosis of individual muscles could be found.³⁷ Both antibody-mediated or *myostatin* propeptide-mediated myostatin blockade in mdx mice increased muscle strength and reduced the dystrophic pathophysiology.³⁸ The genetic inactivation of *myostatin* in muscle-derived stem cells (MDSC) was associated with the silencing of critical genes for early myogenesis. Skeletal myogenesis-related genes, e.g., *MyoD1* and *Myf5* are downregulated in MDSCs from *myostatin* knock-out mice compared to controls. For this reason, MDSCs from myostatin knock-out mice showed multipotent non-myogenic differentiation but no myogenic differentiation.³⁹ An increase in myostatin in necrotic muscle tissue and its significant reduction in regenerating myogenic cells could also be proven.⁴⁰ Compared to controls, a significantly reduced *MSTN* expression could have recently been observed in the masticatory muscles of 100-day-old dystrophic mice.²¹ In the complete regenerated muscle in the mdx mouse after 9 weeks of birth, there was no evidence of myostatin.⁴⁰ Based on our findings, one can act on the assumption that regenerative processes in the masticatory muscles of the mdx mouse after BTX-A injection in the right masseter muscle were highly activated in these mice, which usually cannot be found at this age. In this regard, our study will contribute to a better understanding of regenerative processes at a molecular level in healthy and dystrophic mice muscles.

Conclusions

This study for the first time demonstrated that an intramuscular BTX-A injection into the masseter muscle induced changes in the gene and protein expression of myogenin, myostatin and MyoD1 in the masticatory muscles

of both healthy and mdx mice. In comparison to mdx mice, healthy mice showed a faster regeneration process of the paralyzed skeletal muscle; at the same time, however, compensatory effects could be found in the other masticatory muscles in both mice strains. Due to the increase of protein expression of the investigated genes in mdx mice, it can be assumed that the regeneration processes had been evolved much more slowly, which could be associated with the muscle dystrophy. Further investigations are necessary to better understand the time course of regeneration processes in the mdx mouse after BTX-A injection.

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