Influence of Botulinumtoxin A on the Expression of Adult MyHC Isoforms in the Masticatory Muscles in Dystrophin-Deficient Mice (Mdx-Mice)

Ute Ulrike Botzenhart, Constant in Wegenstein, Teodor Todorov, and Christiane Kunert-Keil

Department of Orthodontics, Carl Gustav Carus Campus, TU Dresden, Fetscherstrasse 74, 01307 Dresden, Germany

Correspondence should be addressed to Ute Ulrike Botzenhart; ute.botzenhart@uniklinikum-dresden.de

Received 29 April 2016; Revised 27 June 2016; Accepted 10 July 2016

Academic Editor: Patrizia Rovere-Querini

Copyright © 2016 Ute Ulrike Botzenhart et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

The most widespread animal model to investigate Duchenne muscular dystrophy is the mdx-mouse. In contrast to humans, phases of muscle degeneration are replaced by regeneration processes; hence there is only a restricted time slot for research. The aim of the study was to investigate if an intramuscular injection of BTX-A is able to break down muscle regeneration and has direct implications on the gene expression of myosin heavy chains in the corresponding treated and untreated muscles. Therefore, paralysis of the right masseter muscle was induced in adult healthy and dystrophic mice by a specific intramuscular injection of BTX-A. After 21 days the mRNA expression and protein content of MyHC isoforms of the right and left masseter, temporal, and the tongue muscle were determined using quantitative RT-PCR and Western blot technique. MyHC-IIa and MyHC-I-mRNA expression significantly increased in the paralyzed masseter muscle of control-mice, whereas MyHC-IIb and MyHC-IIx/d-mRNA were decreased. In dystrophic muscles no effect of BTX-A could be detected at the level of MyHC. This study suggests that BTX-A injection is a suitable method to simulate DMD-pathogenesis in healthy mice but further investigations are necessary to fully analyse the BTX-A effect and to generate sustained muscular atrophy in mdx-mice.

1. Introduction

The stomatognathic system is based on a close mutual and functional network of different hard and soft tissues from those the masticatory muscles illustrate as an essential component. They are one of the strongest muscles of the body [1].

In mammals, muscle contraction is possible due to highly organized motor units consisting of a motor neurone located in the brain stem [2], its axons, and a colony of corresponding fibres [2, 3]. Motor units show a large variability in morphological and physiological characteristics [2] and can be distinguished on the basis of the differences in contraction time, twitch tension, susceptibility to fatigue, and histochemical staining [3]. Three classes of motor units called slow fatigue resistant, fast fatigable, and fast fatigue resistant were initially identified in mammalian skeletal muscles composed by slow oxidative type I and fast glycolytic type IIa and IIb fibres, respectively [3–5]. A fourth motor unit type with fast contractile characteristics and intermediate fatigability composed by type IIx fibres was subsequently detected in rat skeletal muscles [6, 7]. Due to that fact, the most informative methods to delineate muscle fibre types are based on specific myosin profiles, especially the myosin heavy chain isofrom complement [8] possibly being more related to functional behaviour of jaw muscle motor units than past histochemical classifications [6].

Myosin heavy chains exist in multiple isoforms that are differentially distributed in the various fibre types [9]. At least, four different isoforms of myosin heavy chains are expressed in adult skeletal muscle: slow isoform type I coded by Myh7 gene [10] and fast isoforms type IIa, IIx/d, and IIb [8, 11–14] coded by Myh2, Myh1, and Myh4, respectively [10]. The distribution of different MHCs and fibre types varies in
a muscle-specific as well as a species-specific manner [3, 15] and depending on the function, the anatomical location, and structure of the muscle [3, 12, 14]. For example, limb muscles predominantly contain type I fibres [3]. In the orofacial muscles, especially the masseter muscle, a different fibre distribution has been reported showing a wide variation in fibre type composition as demonstrated in biopsy studies [16]. Presumably depending on functional partitioning of activity of this muscle [6], a predominance of type I fibres in the anterior part and a general presence of hybrid fibres have been described as a normal feature of this muscle in humans [17, 18]. For temporal muscle a predominance of type I fibres in the anterior part (46%) [19] and lower portion of type I fibres (24%) in the posterior part may be also due to functional compartmenting.

Muscle fibres are versatile and dynamic entities capable of adjusting their phenotypic properties under various conditions and in response to altered functional demands and display a great adaptive potential [8, 20]. The dynamic nature of skeletal muscle fibres is achieved by the adaption of its MyHCs composition [1, 21], and changes in its expression result in fibre type transitions [8]. This process can be regarded as a significant contribution to improve survival [8]. In developing muscles, fibre composition of individual muscle groups varies dramatically [12], fibre transitions is usually seen, and MyHCs expression is regulated by neural, hormonal, mechanical, and other factors [9, 22, 23], but transitions of fibre types can also be found in adult muscle fibres due to biological ageing [2, 8, 24], activation intensity [2], neuromuscular activity [8, 25] or electrical stimulation [26, 27], hormone levels [2, 8, 28], exercises [29–32], training, during muscle atrophy induced by denervation [33], physical damage, and also muscle disease [34].

Duchenne muscular dystrophy (DMD), a X-linked recessive disease [35, 36], is one of the most common and most serious myopathies which affects approximately 1:3500 live born males [36, 37]. Its onset is between 2 and 5 years of age [35] with clinical symptoms of muscle weakness in the limbs and pseudohypertrophy of calf muscles [38, 39]. At the age of 10–12 years, due to the loss of ambulation, the patients are wheelchair bound [40, 41] and usually the onset of masticatory involvement comes along with that time span. Interestingly the orofacial muscles are affected approximately 2 years later [42], which results in muscle imbalance and severe craniofacial and dental abnormalities [42–44].

The disease is caused by mutations in the dystrophin gene [35], encoding for the sarcolemmal protein product dystrophin, which is part of the DGC (dystrophin glycoprotein complex) and links the motor protein actin to the cytoskeleton [45]. It thus provides mechanical stability to striated muscles [46]. Absence of dystrophin leads to disintegration of the DGC, instability of muscle cell membrane, uncontrolled calcium influx [47], sterile inflammation, and progressive muscle degeneration [35, 48].

Currently the best-characterized and most widely used [49] animal model for DMD is the naturally occurring dystrophin-deficient mdx-mouse [50, 51], which carries a null mutation in exon 23 of the dystrophin gene [35, 47, 49]. Mdx-mice are a genetically biochemical homologue of the disease [37] but show a relative mild pathology [47], and severity of the phenotype is less than that in the human condition [36, 52]. Mdx-mice display hallmark symptoms of the diseases, changes in skeletal muscle histology, respiratory insufficiency, cardiomyopathy, and muscle weakness [36].

Botulinum toxin A (BTX-A), a neurotoxin that blocks the release of acetylcholine at the neuromuscular junction and thus muscle contraction [53, 54] may lead to loss of muscle function caused by muscle atrophy [55, 56]. The therapeutic effects of BTX-A first appear in 1 to 3 days, peak in 1 to 4 weeks, and decline after 3 to 4 months [57].

Due to the fact that the mdx-mouse shows a higher regenerative capacity that ensures a more benign phenotype and essentially normal function [58], there is only a restricted time slot for research. To extend the time frame for evaluation of muscle influence on craniofacial growth and deformities, the purpose of this study was to determine the effects of BTX-A injection in the right masseter muscle of healthy and mdx-mice on the MyHC expression and protein content, to draw conclusions concerning remodelling and adaption of the injected muscle as well as the adjacent noninjected masseter, temporal, and tongue muscles.

2. Materials and Methods

In this study the experimental protocol and all procedures were approved by the Laboratory Animal Research Committee of Saxony with the number 24-9168.11-1/2013-46.

2.1. Animals. As subjects mice of the line C57Bl/10ScSn (control group, n = 10) and C57/Bl10ScSn-Dmdy (mdx) (test group, n = 10) of both genders, aged 100 days at baseline of the trial and a body weight of approximately 30 g, were used in this study. The mice originally obtained from Jackson Laboratory (Bar Harbor, USA) were born in the laboratory animal experimental centre of Dresden and up to the beginning of the experiments housed in standard cages in groups of 2–6 animals under standardised conditions in a temperature-controlled room of 21–23°C maintained on a 12:12 h light-dark cycle with standard mice chow and water available ad libitum.

2.2. Chemodenervation Using Botulinum toxin A (BTX-A). For chemodenervation the mice were temporally anaesthetized by an intraperitoneal injection consisting of a mixture of 10% ketamine (Ceva, Tiergesundheit GmbH, Düsseldorf, Germany) and 2% Rompun (Bayer, HealthCare AG, Leverkusen, Germany) in a relation to 3:2 at a dose of 0.1 mL per 100 g biomass.

To induce muscle paralysis, one single specific intramuscular injection of 0.025 mL BTX-A (Botox®, Allergan®, Irvine, California, USA; 1.25 IU/0.1 mL in physiologic NaCl-solution) was administered in the superficial and deep venter of the right masseter muscle. Both, healthy and mdx-mice were injected.

Postinjection care included a three-time daily control of the health state of the mice after they had awoken from anaesthesia and the verification of paralysis. The effects
of chemodenervation after BTX-A injection usually start delayed. Paralysis in the BTX-A injection masseter is typically evident by the refusal of solid food and tooth chattering 3 days after injection. Due to that fact, the mice were kept under standard conditions described above with the possibility to choose between soft or solid food for the first 7 days. After that time, only solid food was offered. Until the terminal experiments the weight of the mice was controlled regularly.

2.3. Muscle Sample Preparation and Processing. 21 days after intramuscular BTX-A injection, the animals were painlessly killed accordingly of the international guidelines for animal protection by means of an overdose of Isoflurane. The head was immediately separated from the body and the following muscles were carefully dissected and harvested by the same trained operator: masseter muscle (superficial venter) and temporal muscle from the injection side and contralateral side, respectively, as well as the tongue muscle.

According to Baverstock et al. [59], the dissected masseter muscle tissue corresponded to the superficial and (in parts) the deep masseter, accounting for 19% and 33% of overall masticatory muscle mass, respectively. The dissected temporal muscle, usually described as consisting of two parts and in the mouse distinguished as lateral and medial part, corresponded to the medial temporal muscle, which accounts for 77% of overall temporal muscle mass and is the larger portion of both parts [59].

For each of the examined muscles mRNA expression and protein content of myosin heavy chain (MyHC) isoforms were assessed. In addition, directly after dissection the right and left masseter muscles were weighted with a precision balance for direct comparison of muscle wet mass of the injected as well as the untreated contralateral side. The muscles were then flash-frozen in liquid nitrogen at −173°C and up to subsequent analysis stored in a deep fridge at −80°C.

2.4. RNA-Extraction and Reverse Transcription Reaction. Total RNA isolation from the muscle tissues was performed using Trizol (QIAzol Lysis Reagent QIAGEN, Hilden, Germany) and the RNeasy® Mini Kit (QIAGEN, Hilden, Germany) according to manufacturer’s instructions. RNA concentrations and purity of the eluate were determined photometrically by ultraviolet absorbance measurements in the Eppendorf BioPhotometer® (Eppendorf Vertrieb Deutschland GmbH, Wesseling-Berzdorf, Germany) at a wavelength of 260 nm, a background compensation for the absorbance at 280 nm, and distilled water for calibration. Reverse transcription for synthesis of cDNA was performed in the Thermocycler TOptical (Analytik Jena AG, Jena, Germany) with 200 ng RNA and the innuSCRIPT Reverse Transcriptase in Nucleotide Mix and Random primer (Analytik Jena AG) following manufacturer’s protocol.

2.5. qRT-PCR (Quantitative Real-Time Polymerase Chain Reaction). The mRNA quantification of four different myosin heavy chain isoforms (Myh1, Myh2, Myh4, and Myh7) in the concerning muscle tissues was performed by qRT-PCR (Taq-Man® Assays; PE Applied Biosystems®, Weiterstadt, Germany) in comparison to 18S mRNA (Eucaryotic 18S rRNA Endogenous control: 4310893E; PE Applied Biosystems®, Weiterstadt, Germany) using gene-specific TaqMan PCR probes and primers which have been previously described elsewhere [60]. The master mix contained innuMix qPCR Master Mix Probe (Analytik Jena AG), 10x specific probes and primers, and RNase free water. For quantification of gene expression of the different MyHCs, 8 ng cDNA was used in a final volume of 20 μL. Gene amplification was performed with the TOptical cycler (Analytik Jena AG) at 95°C for 10 minutes for initial denaturation followed by 40 cycles, in each case 10 seconds at 95°C and 45 seconds at 60°C. Absolute copy numbers of the studied genes and 18S cDNA were determined using calibration curves generated with cloned PCR fragment standards [60]. Copy numbers of individual transcripts were given in relation to those of 18S cDNA. Each probe was performed twice and a “nontemplate control” was carried out parallel in all experiments to validate results.

2.6. Western Blot. To extract muscle protein, the interphase and phenophase from the RNA isolation protocol were used. Protein isolation was performed following the manufacturer’s protocol (isolation of DNA and protein from QIAzol Reagent-lysed samples (RY16 May-04) (QIAGEN, Hilden, Germany)). To identify the MyHC isoforms, 15 μg of proteins from each muscle was loaded onto Citerion™ TGX Stain-free™ Precast Gels (Bio-RAD Laboratories GmbH, Munich, Germany) and separated by 100 V (constant Voltage) for 60 minutes. Following the electrophoresis, the proteins were transferred to the Western blot membrane, using the Trans-Blot® Semi-Dry transfer system (Trans-Blot® Turbo™ Mid PVDF Transfer Packs; Bio-RAD Laboratories GmbH) and the Trans-Blot Turbo blotting apparatus (Bio-RAD Laboratories GmbH). After protein transfer to the PVDF membrane, membranes were blocked over night at 4°C with 5% dry milk in phosphate-buffered saline (PBS) buffer with Tween.

Blots were incubated with monoclonal antibodies against MyHC proteins (anti-fast skeletal myosin antibody [My-32] (1:1000; Abcam, Cambridge, UK) and monoclonal Anti-Myosin (Skeletal, Slow) antibody produced in mouse (clone NOQ 7.5.4D; 1:1000; Sigma-Aldrich GmbH, Munich, Germany) diluted in PBS containing 5% dry milk and 0.025% NaN₃ for 2-3 h at room temperature. Horseradish peroxidase (HRP-) conjugate goat anti-mouse immunoglobulin (1:5000; Dako, Hamburg, Germany) were used afterwards. Bound antibodies were detected and visualized using an enhanced chemiluminescence system (WesternBright Chemilumineszenz Substrat Quantum, Advanta Inc., Menlo Park, USA). On each gel monoclonal anti-glyceraldehyde-phosphate dehydrogenase (GAPDH) antibody (clone 6C5; 1:1000; Millipore, Billerica, Massachusetts, USA) served as loading control of the gels to finally calculate the protein content (incubation at room temperature for 2 h). Quantitative analyses of protein bands from MyHC isoforms and GAPDH in the examined masticatory muscle of the 100-day-old healthy and mdx-mice were undertaken using GelScan 5.2 software (Serva, Heidelberg, Germany). Mean optical
density ± SEM are given in all cases for n = 4 muscle samples of different animals and three-independent Western blot analysis.

2.7. Statistics. To evaluate differences in the mRNA expression and protein content of the different fibre types in the analysed masticatory muscles of healthy and mdx-mice, statistical analysis was performed using the Software SigmaStat® Version 3.5 (Systat Software Inc., San Jose, California, USA) and Mann Whitney U test. In case of a normal distribution, the t-test was applied. Results p ≤ 0.05 were regarded as statistically significant.

3. Results

3.1. Body Weight and Muscle Dimensions. An initial decrease in body weight in the very early postinjection days, which might be attributed to the beginning paralysis and reduced food ingestion, was followed by a steady increase during the experimental protocol up to its closure. A statistical difference in body weight between the control and mdx group was not obvious at any time. During the entire experimental setup the body weight was similar in both groups.

To verify BTX-A induced atrophy, muscle mass of the injected masseter muscle and the corresponding contralateral noninjected muscle was measured. A comparison of the muscle tissue mass revealed a statistically significant difference between the weight and size of the injected (right) and untreated (left) masseter muscles for both control and mdx-mice (Figure 1). Compared to the untreated left masseter muscles, injected right masseter muscles were 56% and 45% smaller in the control and mdx group, respectively.

3.2. mRNA Expression of MyHC Isoforms. At first, the already known expression difference between dystrophic and healthy mice was also detectable, such as a decrease of Myh2 mRNA and an increase of Myh4 mRNA in dystrophic masseter muscle as well as an increase of Myh7 mRNA expression levels (encoding for fibre type I) in dystrophic temporal muscle compared to control.

21 days after BTX-A injection in the right masseter muscle, mRNA expression of MyHCs in the extracted muscle tissues of injected atrophic masseter muscle of control-mice differed significantly from the untreated noninjection side. A significant decrease of Myh4 mRNA and Myh1 mRNA encoding for the fast fibre types IIb and IIXd, respectively, could be detected, whereas Myh2 and Myh7 mRNA expression, corresponding to the fibre types IIA and I, were significantly increased. In control temporal muscle tissue of healthy mice no differences between the right and left side were found. In tongue muscle a high expression level of Myh4 mRNA in the control as well as mdx group, with no statistically significant differences between both groups, could be detected.

The mRNA expression of all tested MyHC isoforms remained unchanged in mdx muscle tissue from both sides. No differences between BTX-A treated and untreated masseter muscle as well as right and left side could be observed in this mice strain (Table 1).

3.3. Protein Content. Semiquantitative analysis of fast and slow skeletal myosin proteins were performed with Western blot analysis. Single immune-reactive bands of approximately 220kDa were detected. These bands correspond to the expected molecular weights of slow as well as fast myosin, respectively (Figure 2(a)). The quantitative evaluation of Western blots only showed significant increased protein levels of MyHC-II in BTX-A treated control masseter muscle compared to contralateral untreated masseter muscle tissue of control-mice (p = 0.006; Figure 2(c)). In neither other muscle samples from control-mice nor all muscle samples from dystrophic mice differences in the expression levels of fast and slow myosin heavy chains could be detected (Figure 2).

4. Discussion

In the present study the effects of different masticatory muscles after BTX-A injection in the right masseter of healthy and mdx-mice muscles were evaluated concerning mRNA and protein expression of MyHC isoforms. BTX-A was used to reduce masticatory function and to prolong dystrophic features of muscle fibres in the masseter muscle of mdx-mice. Usually muscle pathology of mdx-mice is most pronounced and peaks at the age of 3-4 weeks [35, 61] and goes to cycles of de- and regeneration by 9–12 weeks of age, where regeneration overcomes degeneration of muscle fibres [62, 63]. In our study 100-day-old control and dystrophic mice were used. It has previously been shown that muscle fibres of mdx-mice of this age have centralized nuclei in 75% of all muscle fibres, a typical sign of muscle fibre regeneration [64].

BTX-A injection and inactivation of the right masseter muscle clearly resulted in a reduction of muscle weight 21 days after injection. It is well known that BTX-A treatment indirectly induces masticatory hypofunction and muscle
Table 1: mRNA expression of MyHC isoforms (Myh1, Myh2, Myh4, and Myh7) in BTX-A treated and untreated muscle tissue, 21 days after injection into the right masseter muscle of 100-day-old mice (control/mdx; \( n = 10 \) each). Means ± standard deviations; Student's \( t \)-test \( ^* p \leq 0.05 \) treated versus untreated masseter muscle; \( ^# p \leq 0.05 \) control versus mdx. Significant values are indicated by bold lettering.

| Gene name | Fibre type | Masseter right | Temporal right | Masseter left | Temporal left | Tongue |
|-----------|------------|----------------|----------------|---------------|---------------|--------|
|           |            | Control Mdx    | Control Mdx    | Control Mdx   | Control Mdx   |        |
| Myh1      | IIx/d      | 1.06 ± 0.71 \( ^* p = 0.041 \) | 1.37 ± 0.64 | 1.63 ± 0.87 | 1.19 ± 0.36 | 1.27 ± 0.88 | 1.14 ± 0.67 | 1.68 ± 1.35 | 1.82 ± 1.2 | 0.23 ± 0.16 | 0.63 ± 0.67 |
| Myh2      | IIa        | 2.29 ± 0.79 \( ^* p < 0.001 \) | 1.23 ± 0.64 | 0.77 ± 0.59 | 0.75 ± 0.45 | 4.41 ± 2.97 | 3.41 ± 0.54 | 3.67 ± 1.85 | 4.37 ± 3.05 | 0.02 ± 0.02 | 0.07 ± 0.09 |
| Myh4      | IIb        | 1.18 ± 0.90 \( ^* p = 0.023 \) | 2.63 ± 1.57 | 2.99 ± 2.43 | 2.60 ± 1.85 | 9.36 ± 6.66 | 9.80 ± 8.79 | 19.47 ± 17.15 | 10.12 ± 12.48 | 25.8 ± 17.71 | 33.02 ± 17.29 |
| Myh7      | I          | 0.000133 ± 8.3E6 \( ^* p = 0.012 \) | 0.000041 ± 3.3E6 | 0.000053 ± 8.9E6 | 0.000151 ± 2.9E5 | 0.000215 ± 3.7E5 | 0.000113 ± 1.8E5 | 0.000261 ± 7.2E5 | 0.000005 ± 2.3E6 | 0.000001 ± 6.0E6 |
atrophy due to chemoinactivation [53]. Changes in muscle weight after BTX-A injection could also previously be found [65] and marked atrophy of paralyzed muscle fibres [5, 55] is a common feature also seen under clinical conditions taking advantage of BTX-A treatment for masseter hypertrophy [66, 67] and bruxism [68, 69]. In animal experiments, the muscle fibres begin to show atrophy histologically within 10–14 days after injection and this atrophy continues to develop over 4–6 weeks [70]. As shown previously, BTX-A influences the expression of MyHC isoforms. After BTX-A injection into the right masseter muscle of pigs a fibre shift towards faster isoforms in the injected as well as to slower isoforms in the noninjected masseter muscle could be demonstrated [71]. Slow-to-fast fibre shift is also well known from other denervation studies where no nerve stimulation exists [5]. In our study at protein level similar effects could be seen in injected control masseter muscle with a statistically significant increase of type II myofibres compared to the contralateral noninjection side, whereas treated dystrophic masseter muscle does not show any statistically significant differences compared to untreated masseter muscle of mdx-mice.

By contrast, at mRNA level some other effects have been demonstrated in wild-type mice. In the right masseter muscle of control-mice a decrease in MyHC-IIb and -IIx mRNA expression as well as an increase in MyHC-IIa encoding for the slowest form of type II myofibres and an increase of MyHC-I encoding for slow muscle fibres could be identified. It is well known that healthy mouse masseter muscle (superficial part) predominantly consists of fast type IIb myofibres [72, 73]. A reduction of fast muscle fibres is typically seen in dystrophic muscles. Petrof et al. have proved preferential
degeneration of type II fibres in mdx limb and trunk muscles [74] and in dystrophic masticatory muscles reduced MyHC-IIb mRNA expression was also detected [60]. Furthermore, it was reported that the IIb fibres degenerate first in DMD patients [75]. Analyses of MHC isoforms in the affected diaphragm of canine X-linked muscular dystrophy (CXMD1) also indicated a marked increase of type I and decrease of type IIa myosin isoforms [76]. In histological studies a fibre shift with more pronounced portion of slow type I fibres has been described for the superficial masseter muscle as a hallmark of disease progression obvious in 14-week-old mdx-mice [77]. Lee et al. by their study indicated that after degeneration the regenerated muscle acquires muscle fibre characteristics entirely different from those in its normal counterpart, with a very strong expression of MyHC-I in mdx-mouse masseter at 9 weeks of age [63]. The MyHC-IIX isoform has already been reported to represent the transition from fast type II to slow type I myofibres [22] and subsequently this transition results in a decrease of type II fibres. Based on these findings, it is to be assumed that chemical denervation, induced by BTX-A, simulates dystrophic appearance in healthy mice masticatory muscles with regard to fibre composition.

Muscles can also induce a fibre shift towards slower isoforms to adapt to varying functional load and endurance training [78]. In 10-week-old pigs, after chronic sagittal advancement of the mandible, a significant increase in the cross-sectional area of type I fibres and type I MyHC expression in the anterior part of the masseter, distal part of the temporal and the medial pterygoid muscle could be shown, which was simultaneously accompanied by a comparable decrease in type II MyHCs in these muscles [21]. Step-by-step transition of muscle fibres from type IIB via type IIA to type I under activity was also found by Goldspink [79] and Pette and Staron [80] and they stated that under higher muscle stress the increase in cross-sectional area of type I fibres is more efficient for long-term energy accumulation. In our study, in healthy mice after BTX-A injection a decrease in fast muscle fibres was observed in the untreated masseter muscle, which was statistically significantly different from the injection side showing at once an increase in type II fibres. It is well known that stretch overload influences fibre type transition towards slower MyHC isoform expression [8], and the same transition takes place in case of functional overload on skeletal muscles [81]. In the right temporal muscle of control-mice an increase of MyHC-II protein expression could also be observed, but these results were not statistically significantly different from the contralateral side. However this tendency of type II fibre increase seen could possibly be traced back to a compensatory effect of functional loss of the right masseter muscle and the rapid force increase and high stress, which is known to induce a fibre shift towards faster isoforms [78]. Harzer et al. emphasized that short-time and long-term strain on muscles due to endurance and fast-force training lead to different reactions, respectively. Though endurance training promotes a fibre shift from fast type II muscle fibres to slow type I with a more efficient energy supply during constant load [82], whereas fast-force training promotes a fibre shift in the opposite direction [78]. It can be assumed that due to the BTX-A induced inactivation of the right masseter muscle, by simultaneous preservation of the chewing function, other muscle groups might have experienced more stress and might have adapted by changing their fibre type composition. Nevertheless this effect was only significant for the left masseter muscle in control-mice. On the other hand the effect seen at protein level in healthy mice could also be explained by BTX-A induced fibre shift towards faster isoforms in the injected muscle tissue whereas fibre type composition of left masseter muscle might not have been changed. This explanation seems to be more suitable because at protein level a statistically significant increase of type I fibres in left masseter muscle tissue was missing.

In contrast to healthy control-mice, muscles of mdx-mice showed no remarkable changes induced by BTX-A injection. It had been expected that the dystrophic injected masseter muscle had been reacted in the same way the healthy muscle did, but at protein level a statistically significant shift towards faster isoforms could not be seen in this muscle although such a tendency could be found. One explanation could be that the regeneration potential of this muscle was already exhausted due to the disease, and a shift towards faster isoforms could not be realized. Another possible explanation is due the role of dystrophin, which attributes particular importance in the expression of type IIB fibres. Webster et al. already emphasized that fast fibres are preferentially affected in DMD, and it has been suggested that dystrophin gene product plays a specific and essential role in IIB fibre function, a subpopulation of muscle fibres specialized to respond to the highest frequency of neuronal stimulation with maximal rates of contraction [75]. Possibly dystrophin is essential for accepting the high-frequency activity of fast motor neurons to carry out the high-frequency contraction demanded for type IIB muscle fibres [75]. In this case, the defect in the muscle fibre would be manifested only under neural influence [83]. Hence, with innervation being absent no degeneration of IIB fibres should occur. Vice versa, even though with innervation and strong IIB mRNA expression being present, which could be seen on mRNA level in our study, on account of dystrophin deficiency, no translation for MyHC-IIB exists. So that the last mentioned explanation is more appropriate to explain our results. Perhaps by this reason the reaction to BTX-A injection occurs temporally delayed in this mouse strain. Usually mRNA expression goes ahead to protein expression which occurs as time-delayed. In this context the fact that only one time point was investigated needs to be considered critically. On the other hand, it was of special interest to investigate both protein and mRNA expression in the same muscle tissues after BTX-A injection to estimate changes occurring at exactly one time point during the process of muscle fibre adaption. The mRNA response to stress is very rapid; thus mRNA content represents a steady state of muscle adaption, but with the additional analysis of protein content more distinctive effects of BTX-A injection could be demonstrated.

Effects on mRNA expression encoding for MyHC isoforms found in injected muscles in our study, especially in healthy mice, could also be attributed to reinnervation process which is known to occur 3-4 weeks after denervation [84]. Nayyar et al. stated that after that time, at the
cellular level, upregulation of the muscle nicotinic receptors and accordingly reappearance of muscle innervation, due to sprouting could be found in mice after a single BTX-A injection [84]. Hence, the results presented here may reflect the sum of fibre shifts in specific masticatory muscles evoked by BTX-A induced immobilisation and subsequent recovery of muscle function, reflected by fibre type diversity in different muscles and accumulation of specific types during fibre shift to one end of the fibre spectrum.

In conclusion, our study confirmed that a fibre shift due to BTX-A injection is possible in healthy mice muscle tissue, inducing different fibre type transformations. Likewise, the mRNA MyHC expression demonstrated similar changes to those observed in the untreated dystrophic muscles. However, in dystrophic mice BTX-A injection did elicit neither detectable direct or indirect compensatory fibre shifts, nor a prolongation of the dystrophic phenotype. Further research at different time points is necessary to fully elucidate the impact of BTX-A injection on masticatory muscles and to better estimate whether this medication or treatment method is likely to be able to selectively switch off the active regeneration processes of the musculature in the mdx-mouse and therefore to be able to use this effect for research concerning craniofacial changes induced by functional adaptions.

Competing Interests
The authors declare that they have no competing interests regarding the publication of this paper.

Acknowledgments
The authors like to thank Mrs. Krause for excellent technical assistance.

References
[1] T. Gedrange and W. Harzer, “Muscle influence on postnatal craniofacial development and diagnostics,” Fortschrritte der Kieferorthopädie, vol. 65, no. 6, pp. 451–466, 2004.
[2] T. M. G. J. van Eijden and S. J. J. Turkawski, “Morphology and physiology of masticatory muscle motor units,” Critical Reviews in Oral Biology and Medicine, vol. 12, no. 1, pp. 76–91, 2001.
[3] A. J. McComas, “Oro-facial muscles: internal structure, function and ageing,” Gerodontology, vol. 15, no. 1, pp. 3–14, 1998.
[4] R. E. Burke, D. N. Levine, P. Tsairis, and F. E. Zajac III, “Physiological types and histochemical profiles in motor units of the cat gastrocnemius,” The Journal of Physiology, vol. 234, no. 3, pp. 723–748, 1973.
[5] S. Ciciliot, A. C. Rossi, K. A. Dyar, B. Blauw, and S. Schiaffino, “Muscle type and fiber type specificity in muscle wasting,” The International Journal of Biochemistry & Cell Biology, vol. 45, no. 10, pp. 2191–2199, 2013.
[6] A. G. Hannam and A. S. McMillan, “Internal organization in the human jaw muscles,” Critical Reviews in Oral Biology and Medicine, vol. 5, no. 1, pp. 55–89, 1994.
[7] L. Larsson, L. Edstrom, B. Lindegren, L. Gorza, and S. Schiaffino, “MHC composition and enzyme-histochemical and physiological properties of a novel fast-twitch motor unit type,” The American Journal of Physiology—Cell Physiology, vol. 261, no. 1, pp. C93–C101, 1991.
[8] D. Pette and R. S. Staron, “Myosin isoforms, muscle fiber types, and transitions,” Microscopy Research and Technique, vol. 50, no. 6, pp. 500–509, 2000.
[9] S. Schiaffino and C. Reggiani, “Myosin isoforms in mammalian skeletal muscle,” Journal of Applied Physiology, vol. 77, no. 2, pp. 493–501, 1994.
[10] S. Schiaffino and C. Reggiani, “Fiber types in mammalian skeletal muscles,” Physiological Reviews, vol. 91, no. 4, pp. 1447–1531, 2011.
[11] J. J. Sciote and T. J. Morris, “Skeletal muscle function and fibre types: the relationship between occlusal function and the phenotype of jaw-closing muscles in human,” Journal of Orthodontics, vol. 27, no. 1, pp. 15–30, 2000.
[12] A. Weiss and L. A. Leinwand, “The mammalian myosin heavy chain gene family,” Annual Review of Cell and Developmental Biology, vol. 12, pp. 417–439, 1996.
[13] M. Maejima, S. Abe, K. Sakiyama et al., “Changes in the properties of mouse tongue muscle fibres before and after weaning,” Archives of Oral Biology, vol. 50, no. 12, pp. 988–993, 2005.
[14] T. Gedrange and W. Harzer, “Muscle influence on postnatal craniofacial development and diagnostics,” Fortschrritte der Kieferorthopädie, vol. 65, no. 6, pp. 451–466, 2004.
[15] N. Hamalainen and D. Pette, “Patterns of myosin isoforms in mammalian skeletal muscle fibres,” Microscopy Research and Technique, vol. 30, no. 5, pp. 381–389, 1995.
[16] J. J. Sciote, A. M. Rowlanders, C. Hopper, and N. P. Hunt, “Fibre type classification and myosin isoforms in the human masseter muscle,” Journal of the Neurological Sciences, vol. 126, no. 1, pp. 15–24, 1994.
[17] M. Ringqvist, “Fiber types in human masticatory muscles. Relation to function,” Scandinavian Journal of Dental Research, vol. 82, no. 4, pp. 333–355, 1974.
[18] F. Yu, P. Stål, L. E. Thorneell, and L. Larsson, “Human single masseter muscle fibers contain unique combinations of myosin and myosin binding protein C isoforms,” Journal of Muscle Research and Cell Motility, vol. 23, no. 4, pp. 317–326, 2002.
[19] J. A. M. Korfage and T. M. G. J. Van Eijden, “Regional differences in fibre type composition in the human temporalis muscle,” Journal of Anatomy, vol. 194, part 3, pp. 355–362, 1999.
[20] W. A. Weij, “Masticatory muscles. Part II. Functional properties of the masticatory muscle fibers,” Nederlands tijdschrift voor tandheelkunde, vol. 104, no. 6, pp. 210–213, 1997.
[21] T. Gedrange, A. Lupp, C. Kirsch, W. Harzer, and R. Klinger, “Influence of the sagittal advancement of mandibulae on myofibrillar ATPase activity and myosin heavy chain content in the masticatory muscles of pigs,” Folia Histochemica et Cytobiologica, vol. 40, no. 3, pp. 277–284, 2002.
[22] S. Schiaffino and C. Reggiani, “Molecular diversity of myofibrillar proteins: gene regulation and functional significance,” Physiological Reviews, vol. 76, no. 2, pp. 371–423, 1996.
[23] P. Gunning and E. Hardeman, “Multiple mechanisms regulate muscle fiber diversity,” The FASEB Journal, vol. 5, no. 15, pp. 3064–3070, 1991.
[24] L. Larsson, T. Anved, L. Edstrom, L. Gorza, and S. Schiaffino, “Effects of age on physiological, immunohistochemical and biochemical properties of fast-twitch single motor units in the rat,” The Journal of Physiology, vol. 443, pp. 257–275, 1991.
[25] D. Pette and G. Vrbova, “Invited review: neural control of adaptive properties of myofibrillar expression in mammalian muscle fibers,” *Muscle and Nerve*, vol. 8, no. 8, pp. 676–689, 1985.

[26] D. Pette and G. Vrbová, “Adaptation of mammalian skeletal muscle fibers to chronic electrical stimulation,” *Reviews of Physiology Biochemistry and Pharmacology*, vol. 120, pp. 115–202, 1992.

[27] A. Maier, L. Gorza, S. Schiaffino, and D. Pette, “A combined histochemical and immunohistochemical study on the dynamics of fast-to-slow fiber transformation in chronically stimulated rabbit muscle,” *Cell and Tissue Research*, vol. 254, no. 1, pp. 59–68, 1988.

[28] T. Soukup and I. Jirmánová, “Regulation of myosin expression in developing and regenerating extrafusal and intrafusal muscle fibers with special emphasis on the role of thyroid hormones,” *Physiological Research*, vol. 49, no. 6, pp. 617–633, 2000.

[29] R. R. Roy, K. M. Baldwin, and V. R. Edgerton, “The plasticity of muscle fibers to chronic electrical stimulation,” *Molecular biology and medicine*, vol. 15, no. 5, pp. 517–534, 1994.

[30] S. Schiaffino and G. Salvetti, “Molecular diversity of myofibrillar proteins: isoforms analysis at the protein and mRNA level,” *Methods in Cell Biology*, vol. 52, pp. 349–369, 1997.

[31] H. Klatigaard, O. Bergman, R. Betto et al., “Co-existence of myosin heavy chain I and Ia isoforms in human skeletal muscle fibers with endurance training,” *Pflügers Archiv: European Journal of Physiology*, vol. 416, no. 4, pp. 470–472, 1990.

[32] M. F. Patterson, G. M. M. Stephenson, and D. G. Stephenson, “Denervation produces different single fiber phenotypes in fast- and slow-twitch hindlimb muscles of the rat,” *American Journal of Physiology—Cell Physiology*, vol. 291, no. 3, pp. C518–C528, 2006.

[33] D. F. Goldspink, “The influence of activity on muscle size and protein turnover,” *The Journal of Physiology*, vol. 254, no. 1, pp. 283–296, 1977.

[34] A. Nakamura and S. Takeda, “Mammalian models of duchenne muscular dystrophy: pathological characteristics and therapeutic applications,” *Journal of Biomedicine and Biotechnology*, vol. 2011, Article ID 843939, 8 pages, 2011.

[35] J. Manning and D. O’Malley, “What has the mdx mouse model of duchenne muscular dystrophy contributed to our understanding of this disease?” *Journal of Muscle Research and Cell Motility*, vol. 36, no. 2, pp. 155–167, 2015.

[36] C. A. Collins and J. E. Morgan, “Duchenne’s muscular dystrophy: animal models used to investigate pathogenesis and develop therapeutic strategies,” *International Journal of Experimental Pathology*, vol. 84, no. 4, pp. 165–172, 2003.

[37] V. Jay and J. Vajsar, “The dystrophy of Duchenne,” *The Lancet*, vol. 357, no. 9255, pp. 550–552, 2001.

[38] A. E. H. Emery, “The muscular dystrophies,” *The Lancet*, vol. 359, no. 9307, pp. 687–695, 2002.

[39] K. R. Wagner, “Approaching a new age in duchenne muscular dystrophy treatment,” *Neurotherapeutics*, vol. 5, no. 4, pp. 583–591, 2008.

[40] D. J. Wells and K. E. Wells, “Gene transfer studies in animals: what do they really tell us about the prospects for gene therapy in DMD?” *Neuromuscular Disorders*, vol. 12, supplement 1, pp. S11–S22, 2002.

[41] L. Eckardt and W. Harzer, “Facial structure and functional findings in patients with progressive muscular dystrophy (Duchenne),” *American Journal of Orthodontics and Dentofacial Orthopedics*, vol. 110, no. 2, pp. 185–190, 1996.

[42] S. Kiliaridis and C. Katsaros, “The effects of myotonic dystrophy and Duchenne muscular dystrophy on the orofacial muscles and dentofacial morphology,” *Acta Odontologica Scandinavica*, vol. 56, no. 6, pp. 369–374, 1998.

[43] C. Morel-Verdebout, S. Botteron, and S. Kiliaridis, “Dentofacial characteristics of growing patients with Duchenne muscular dystrophy: a morphological study,” *European Journal of Orthodontics*, vol. 29, no. 5, pp. 500–507, 2007.

[44] D. C. Górecki, “Dystrophin: the dead calm of a dogma,” *Rare Diseases*, vol. 4, no. 1, article e1135777, 2016.

[45] T. A. Partridge, “The mdx mouse model as a surrogate for Duchenne muscular dystrophy,” *The FEBS Journal*, vol. 280, no. 17, pp. 4177–4186, 2013.

[46] G. R. Coulton, A. N. Curtin, J. E. Morgan, and T. A. Partridge, “The mdx mouse skeletal muscle myopathy: II. Contractile properties,” *Neuropathology and Applied Neurobiology*, vol. 14, no. 4, pp. 299–314, 1988.

[47] G. Bullfield, W. G. Siller, P. A. L. Wight, and K. J. Moore, “X chromosome-linked muscular dystrophy (mdx) in the mouse,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 81, no. 41, pp. 1189–1192, 1984.

[48] P. Huang, X. S. Zhao, M. Fields, R. M. Ransohoff, and L. Zhou, “Imatinib attenuates skeletal muscle dystrophy in mdx mice,” *The FASEB Journal*, vol. 23, no. 8, pp. 2539–2548, 2009.

[49] C. Y. Tsai, Y. C. Lin, B. Su, L. Y. Yang, and W. C. Chiu, “Masseter muscle fibre changes following reduction of masticatory function,” *International Journal of Oral and Maxillofacial Surgery*, vol. 41, no. 5, pp. 394–399, 2012.

[50] P. E. Setler, “Therapeutic use of botulinum toxins: background and history,” *The Clinical Journal of Pain*, vol. 18, no. 6, pp. S119–S124, 2002.

[51] S. L. Dodd, J. Selsby, A. Payne, A. Judge, and C. Dott, “Botulinum neurotoxin type A causes shifts in myosin heavy chain composition in muscle,” *Toxicon*, vol. 46, no. 2, pp. 196–203, 2005.

[52] K. Legerlotz, K. G. Matthews, M. D. Christopher, and H. K. Smith, “Botulinum toxin-induced paralysis leads to slower myosin heavy chain isoform composition and reduced titin content in juvenile rat gastrocnemius muscle,” *Muscle and Nerve*, vol. 39, no. 4, pp. 472–479, 2009.

[53] G. T. Clark, A. Stiles, L. Z. Lockerman, and S. G. Gross, “A critical review of the use of botulinum toxin in orofacial pain disorders,” *Dental Clinics of North America*, vol. 51, no. 1, pp. 245–261, 2007.

[54] V. Nigro and G. Piluso, “Spectrum of muscular dystrophies associated with sarcolemmal-protein genetic defects,” *Biochimica et Biophysica Acta*, vol. 1852, no. 4, pp. 585–593, 2013.
[59] H. Baverstock, N. S. Jeffery, and S. N. Cobb, “The morphology of the mouse masticatory musculature,” Journal of Anatomy, vol. 223, no. 1, pp. 46–60, 2013.

[60] A. Spassov, T. Gredes, T. Gedrange et al., “Differential expression of myosin heavy chain isoforms in the masticatory muscles of dystrophin-deficient mice,” European Journal of Orthodontics, vol. 33, no. 6, pp. 613–619, 2011.

[61] J. K. McGeachie, M. D. Grounds, T. A. Partridge, and J. E. Morgan, “Age-related changes in replication of myogenic cells in mdx mice: quantitative autoradiographic studies,” Journal of the Neurological Sciences, vol. 119, no. 2, pp. 169–179, 1993.

[62] J. X. DiMario, A. Uzman, and R. C. Strohman, “Fiber regeneration is not persistent in dystrophic (mdx) mouse skeletal muscle,” Developmental Biology, vol. 148, no. 1, pp. 314–321, 1991.

[63] W.-H. Lee, S. Abe, H.-J. Kim et al., “Characteristics of muscle fibers reconstituted in the regeneration process of masseter muscle in an mdx mouse model of muscular dystrophy,” Journal of Muscle Research and Cell Motility, vol. 27, no. 3-4, pp. 235–249, 2006.

[64] A. Spassov, T. Gredes, T. Gedrange, S. Lucke, D. Pavlovic, and C. Kunert-Keil, “Histological changes in masticatory muscles of mdx mice,” Archives of Oral Biology, vol. 55, no. 4, pp. 318–324, 2010.

[65] J. D. Porter, S. Strebeck, and N. F. Capra, “Botulinum-induced changes in monkey eyelid muscle. Comparison with changes seen in extracocular muscle,” Archives of Ophthalmology, vol. 109, no. 3, pp. 396–404, 1991.

[66] O. K. Arikian, E. U. Tan, T. Kendi, and C. Koc, “Use of botulinum toxin type A for the treatment of masseteric muscle hypertrophy,” Journal of Otolaryngology, vol. 35, no. 1, pp. 40–43, 2006.

[67] H. J. Kim, K.-W. Yum, S.-S. Lee, M.-S. Heo, and K. Seo, “Effects of botulinum toxin type A on bilateral masseteric hypertrophy evaluated with computed tomographic measurement,” Dermatologic Surgery, vol. 29, no. 5, pp. 484–489, 2003.

[68] E.-K. Tan and J. Jankovic, “Treating severe bruxism with botulinum toxin,” Journal of the American Dental Association, vol. 131, no. 2, pp. 211–216, 2000.

[69] G. E. Borodic, R. J. Ferrante, and L. B. Pearce, “Pharmacology and histology of the therapeutic application of botulinum toxin,” in Therapy with Botulinum Toxin, J. Jankovic and M. Hallett, Eds., p. 119, Marcel Dekker, New York, NY, USA, 1994.

[70] T. Gedrange, T. Gredes, A. Spassov et al., “Histological changes and changes in the myosin mRNA content of the porcine masticatory muscles after masseter treatment with botulinum toxin A,” Clinical Oral Investigations, vol. 17, no. 3, pp. 887–896, 2013.

[71] J. M. Eason, G. A. Schwartz, G. K. Pavlath, and A. W. English, “Sexually dimorphic expression of myosin heavy chains in the adult mouse masseter,” Journal of Applied Physiology, vol. 89, no. 1, pp. 251–258, 2000.

[72] A. Tu xen and S. Kirkeby, “An animal model for human masseter muscle: histochemical characterization of mouse, rat, rabbit, cat, dog, pig, and cow masseter muscle,” Journal of Oral and Maxillofacial Surgery, vol. 48, no. 10, pp. 1063–1067, 1990.

[73] B. J. Petrof, H. H. Stedman, J. B. Shrager, J. Eby, H. L. Sweeney, and A. M. Kelly, “Adaptations in myosin heavy chain expression and contractile function in dystrophic mouse diaphragm,” The American Journal of Physiology, vol. 265, no. 3, pp. C834–C841, 1993.

[74] C. Webster, L. Silberstein, A. P. Hays, and H. M. Blau, “Fast muscle fibers are preferentially affected in Duchenne muscular dystrophy,” Cell, vol. 52, no. 4, pp. 503–513, 1988.

[75] K. Yuasa, A. Nakamura, T. Hiji k ata, and S. Takeda, “Dystrophin deficiency in canine X-linked muscular dystrophy in Japan (CXMD1) alters myosin heavy chain expression profiles in the diaphragm more markedly than in the tibialis cranialis muscle,” BMC Musculoskeletal Disorders, vol. 9, article 1, 2008.

[76] M. Ukai, N. Maeda, and K. Osawa, “Postnatal changes in the extrafusal muscle fiber and muscle spindle in the masseter muscle of the dystrophic mouse,” Biomedical Research, vol. 14, no. 6, pp. 419–425, 1993.

[77] W. Harzer, M. Worm, T. Gredze, M. Schneider, and P. Wolf, “Myosin heavy chain mRNA isoforms in masseter muscle before and after orthognathic surgery,” Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontology, vol. 104, no. 4, pp. 486–490, 2007.

[78] G. Goldspink, “Gene expression in muscle in response to exercise,” Journal of Muscle Research and Cell Motility, vol. 24, no. 2-3, pp. 121–126, 2003.

[79] D. Pette and R. S. Staront, “Mammalian skeletal muscle fiber type transitions,” International Review of Cytology, vol. 170, pp. 143–223, 1997.

[80] S. Banzet, N. Koulmann, H. Sanchez, B. Serrurier, A. Pennequin, and A. X. Bigard, “Muscle gene expression is strongly related to fast-glycolytic phenotype,” Biochemical and Biophysical Research Communications, vol. 353, no. 3, pp. 713–718, 2007.

[81] R. Thayer, J. Collins, E. G. Noble, and A. W. Taylor, “A decade of aerobic endurance training: histological evidence for fibre type transformation,” The Journal of Sports Medicine and Physical Fitness, vol. 40, no. 4, pp. 284–289, 2000.

[82] G. Vrbova, “Duchenne dystrophy viewed as a disturbance of nerve-muscle interactions,” Muscle & Nerve, vol. 6, no. 9, pp. 671–675, 1983.

[83] P. Nayyar, P. Kumar, P. V. Nayyar, and A. Singh, “Botox: broadening the horizon of dentistry,” Journal of Clinical and Diagnostic Research, vol. 8, no. 12, pp. ZE25–ZE29, 2014.