BTX-A Administration to the Target Muscle Affects Forces of All Muscles Within an Intact Compartment and Epimuscular Myofascial Force Transmission

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Measurement of forces of mono- and bi-articular muscles of an entire intact muscle compartment can allow for a comprehensive assessment of the effects of Botulinum toxin type A (BTX-A) both at and beyond the injection site, and in conditions close to those in vivo. The goal was to test the hypotheses that BTX-A affects (1) the forces of not only the injected but also the noninjected muscles of the compartment, and (2) epimuscular myofascial force transmission (EMFT). Two groups of Wistar rats were tested: Control (no BTX-A injected) and BTX (0.1 units of BTX-A were injected exclusively to the mid-belly of TA). Isometric forces were measured simultaneously at the distal tendons of the tibialis anterior (TA) at different lengths, the restrained extensor digitorum longus (EDL) and the extensor hallucis longus (EHL) muscles and at the proximal tendon of EDL. Five days post-injection, BTX-A did affect the total forces of all muscles significantly: (1) The TA force decreased differentially (by 46.6%–55.9%) for most lengths such that a significant negative correlation was found between force reductions and increased muscle length. The maximum TA force decreased by 47.3%. However, the muscle's length range of force production did not change significantly. (2) Distal and proximal EDL forces decreased (on average by 67.8% and 62.9%, respectively). (3) The EHL force also decreased (on average by 9.2%). The passive forces of only the TA showed a significant increase at higher lengths. EMFT effects were shown for the control group: (1) at the shortest TA lengths, the EDL proximo-distal force differences were in favor of the distal force, which was reversed at higher lengths. (2) the EHL force measured at the shortest TA length decreased (by 34%) as a function of TA lengthening. After BTX-A exposure, such EMFT effects disappeared for the EDL, whereas they remained as profound for the EHL. Exposure to BTX-A does affect forces of all muscles operating in an intact compartment. For the BTX-A injected muscle, the reduction in muscle force becomes less pronounced at higher muscle lengths. BTX-A also has effects on EMFT, however, these effects are not uniform within the anterior crural compartment. Decreased forces of the noninjected synergistic muscles suggest the presence of unintended additional effects of BTX-A both for the targeted distal joint and for the nontargeted proximal joint. [DOI: 10.1115/1.4007823]

Keywords: Botulinum toxin type A, muscle force-length characteristics, rat anterior crural compartment, epimuscular myofascial force transmission

Introduction

Botulinum toxin type A (BTX-A) is a chemical denervant that acts at motor nerve endings to block acetylcholine release [1], which causes paralysis of muscle fibers [2], hence, muscle weakness (i.e., decreased ability for force production). Due to its effectiveness in avoiding the development of contractures [3], BTX-A is used widely in patients with cerebral palsy as an alternative treatment to surgery [4–6].

The effects of BTX-A have been widely studied by quantifying the area of paralysis [7], compound muscle action potential [8], and electromyography [9]. However, reports on mechanical parameters, e.g., twitch and tetanic force have been limited to selected muscle lengths or joint positions [e.g., 8,10]. Herzog et al. made a major contribution to filling this gap in the literature by measuring joint torques in a range of joint angles [11–13]. Experiments on the rabbit quadriceps musculature showed that BTX-A causes more pronounced reductions in knee extension torque at more flexed knee positions [13]. This suggests that the effects of BTX-A on muscle forces and increasing muscle length may be negatively correlated.

Recently, Yaraskavitch et al. showed that the force-length characteristics of both injected soleus and noninjected plantaris muscles of the cat are affected by the poison [14]. BTX has been shown to spread through muscle fascia [15], and its effects beyond the injection site are plausible [16]. As these effects may not be confined only to a neighboring muscle, an experimental model that involves measurement of forces of mono- and biarticular muscles of an entire muscle compartment can allow for a comprehensive assessment of the effects of BTX both at and beyond the injection site.

Collagenous connections between adjacent muscles and extramuscular connective tissues such as collagen-reinforced neurovascular tracts and compartmental boundaries provide connections

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between the muscular and nonmuscular structures of an intact compartment [17–19]. These epimuscular connections feature complex mechanical properties. Similar to other connective tissue structures, these connections have nonlinear force–deformation characteristics [20–22]. In addition, they have been shown to be prestrained [23] and to have inhomogeneous mechanical properties (e.g., the proximal parts of the neurovascular tracts of the anterior crural compartment of the rat are stiffer than the distal parts [24]). Previous studies have shown the occurrence of epimuscular myofascial force transmission (EMFT) via these structures [23,25,26]. Such EMFT is characterized by the interplay of stiffness of muscular tissues and epimuscular connections and it is determined by changes in the position of muscle relative to its neighboring structures [e.g., 27,28]. Recently it has been shown that due to such force transmission, the global length changes of human gastrocnemius muscle and local strains within the muscle can be very different and despite its global isometric condition, local and heterogeneous deformations were found also within the soleus muscle [29].

BTX-A exposure changes the stiffness of muscular tissues because it causes paralysis of muscle fibers within parts of the muscle belly [7]. Therefore, compared to the no BTX-A injected condition, identical changes in relative position globally of the muscle-tendon complex is expected to lead locally to different interactions between the muscular tissues and their epimuscular connections. Consequently, the epimuscular connections can operate at different segments of their complex mechanical properties and cause the EMFT mechanism to change. Note that previously, the effects of BTX on the noninjected adjacent muscle were explored after the intactness of the compartment and the connections of the muscles with surrounding structures had been disrupted and the two muscles' forces were not measured simultaneously [14]. Therefore, the effects of BTX-A on the forces of muscles operating in an intact compartment as well as on EMFT mechanism remain unknown.

We hypothesized that BTX-A affects (1) the forces of not only the injected but also the noninjected muscles of an entire intact compartment, and (2) EMFT. The goal of this study was to test these hypotheses. Additionally, it was to assess the existence of a correlation between injected muscle's length and the effects of BTX-A. These goals were addressed by measuring the force-length properties of the injected muscle as well as the isometric forces of the restrained noninjected muscles of the intact anterior crural compartment of the rat simultaneously, and in conditions close to those *in vivo*.

Methods

Surgical and experimental procedures were approved by the Committee on the Ethics of Animal Experimentation at Boğaziçi University. Male Wistar rats were divided into two groups: (1) control ($n = 8$, mean ± SD body mass = 318.5 ± 12.5 g) and (2) BTX ($n = 8$, mean ± SD body mass = 312.5 ± 14.6 g).

After imposing a mild sedation with an intraperitonal dose of 1 mg/kg ketamine, a circular region of approximately 15 mm radius from the center of the knee cap was shaved. The tibialis anterior (TA) muscle was located by palpation when the ankle was in maximal plantar flexion and the knee angle approximated 90 deg. After marking the center of the knee cap, a second marker was placed at a point 10 mm distal to that, along the tibia. The injection location was 5 mm lateral (along the direction normal to the line segment drawn between the two markers) to the second marker and over the TA muscle. All injections were made exclusively into this muscle, to a depth of 3 mm. Note that at the site of injection, the diameter of the TA approximates 5–5.5 mm, whereas the thickness of the skin approximates 0.7–1 mm. Therefore, the injections were made into the superficial half of the TA muscle.

For the BTX group, each 100 unit vial of vacuum dried, botulinum type A neurotoxin complex (BOTOX, Allergan Pharmaceuticals, Ireland) was reconstituted with 0.9% sodium chloride. The animals received a one-time intramuscular BTX-A injection at a total dose of 0.1 units. The injected volume equaled 20 μl. The control group was injected with the same volume of 0.9% saline solution exclusively. All injections were performed 5 days prior to testing. The animals were kept separately until the day of the experiment in standard cages and in a thermally regulated animal care room with a 12 h dark-light cycle.

Surgical Procedures. The animals were anesthetized using an intraperitoneally injected urethane solution (1.2 ml of 12.5% urethane solution/100 g body mass). Additional doses were given if necessary (maximally 0.5 ml). Immediately following the experiments, the animals were sacrificed by the administration of an overdose of urethane solution.

During the surgery and data collection, the animals were kept on a heated pad (Harvard Apparatus, Homoeothermic Blanket Control Unit) to prevent hypothermia. A feedback system utilizing an integrated rectal thermometer allowed for the control of body temperature at 37 °C by adjusting the temperature of the heated pad.

The skin and the biceps femoris muscle of the left hind limb were removed and the anterior crural compartment including the extensor digitorum longus (EDL), the TA, and the extensor hallucis longus (EHL) muscles were exposed. Only a limited distal fasciotomy was performed to remove the retinaculae (i.e., the transverse crural ligament and the crural cruciate ligament). The connective tissues at the muscle bellies within the anterior crural compartment were left intact.

The specific combination of knee joint and ankle angles (120 deg and 100 deg, respectively) was selected as the *reference position*. In the reference position, the four distal tendons of the EDL muscle were tied together using silk thread. Matching markers were placed on the distal tendons of the EDL, the TA and the EHL muscles, as well as on a fixed location on the lower leg. Subsequently, the distal EDL tendon complex as well as the TA and the EHL tendons were cut as distally as possible. The proximal EDL tendon was cut from the femur, with a small piece of the lateral femoral condyle still attached. In order to provide connection to force transducers, Kevlar threads were sutured to (1) the proximal tendon of the EDL muscle (2) the tied distal tendons of the EDL muscle (3) the distal tendon of the TA muscle and (4) the distal tendon of the EHL muscle.

Within the femoral compartment, the sciatic nerve was dissected free of other tissues, during which process all nerve branches to the muscles of that compartment were cut. Subsequently, the sciatic nerve was cut as proximally as possible.

Experimental Setup. The animal was mounted in the experimental setup (Fig. 1). The femur and foot were fixed with metal clamps such that the ankle was in maximal plantar flexion (180 deg) to allow for the free passage of the Kevlar threads to the distal force transducers. The knee angle was set at 120 deg. Each Kevlar thread was connected to a separate force transducer (BLH Electronics Inc., Canton MA). Care was taken to ensure the alignment of the Kevlar threads were in the muscle line of pull. The distal end of the sciatic nerve was placed on a bipolar silver electrode.

Experimental Conditions and Procedure. Room temperature was kept at 26 °C. For the duration of the experiment, muscle and tendon tissues were irrigated regularly by isotonic saline to prevent dehydration.

The distal and proximal tendons of the EDL and the distal tendon of the EHL muscles were kept in their reference positions at all times during the experiment. The isometric TA force was measured at various muscle-tendon complex lengths. Starting at muscle active slack length, the TA length was increased by moving its force transducer (in increments of 1 mm), until it was 2 mm over the length at which the highest TA force was measured. TA muscle-tendon complex length is expressed as deviation ($\Delta l_{mt\ TA}$)

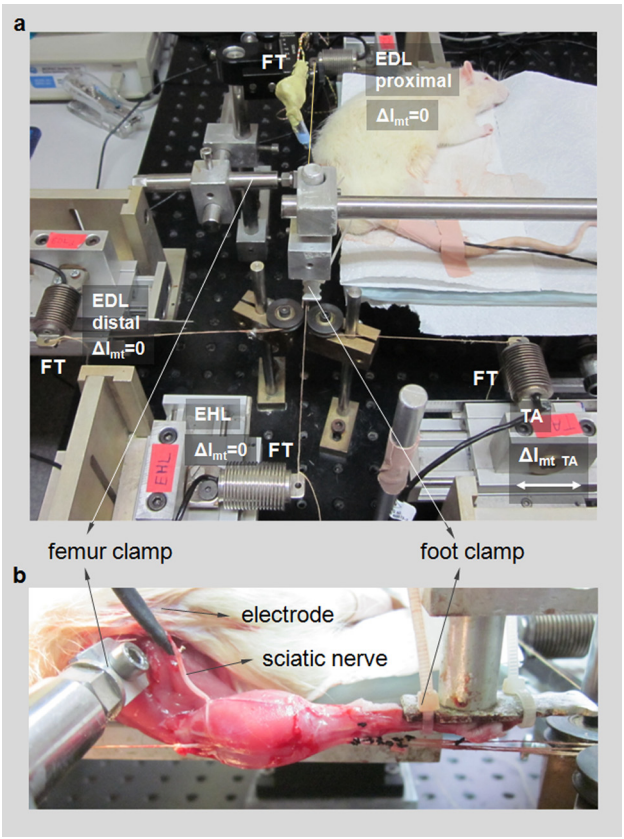

Fig. 1 The experimental setup. (*a*) Distal tendons of the tibialis anterior muscle (TA) and the extensor hallucis longus muscle (EHL) as well as the proximal and the tied distal tendons of the EDL muscle (EDL proximal and EDL distal, respectively) were each connected to a separate force transducer (FT). Throughout the experiment, the EDL and EHL muscles were kept at constant muscle-tendon complex lengths ($\Delta l_{mt} = 0$). Exclusively, the TA muscle was lengthened ($\Delta l_{mt\ TA}$) to progressively increasing lengths, at which isometric contractions were performed. Lengthening (indicated by double arrow) started from muscle active slack length at 1 mm increments by changing the position of the TA force transducer. (*b*) Experimental condition for joint angles: knee angle = 120 deg and the ankle is at maximal plantar flexion. The femur and the foot were fixed by metal clamps and the distal end of the sciatic nerve was placed on a bipolar silver electrode.

from its active slack length. Simultaneously, the proximal and distal EDL forces and the distal EHL force were measured.

All muscles studied were activated maximally by supramaximal stimulation of the sciatic nerve (Biopac Systems stimulator, STMISOC) using a constant current of 2 mA (square pulse width 0.1 ms). After setting the TA muscle to a target length, two twitches were evoked and 300 ms after the second twitch, the muscles were tetanized (pulse train 400 ms, frequency 100 Hz). At 200 ms after the tetanic contraction, another twitch was evoked. After each application of this stimulation protocol, the muscles were allowed to recover for 2 min. For the TA muscle, recovery was allowed to occur near the active slack length, whereas the lengths of the other muscles were not altered.

Processing of Experimental Data and Statistics. Muscle passive isometric forces were determined 100 ms after the second twitch, and muscle total isometric forces were determined during the tetanic plateau (the mean force for a 200 ms interval, 150 ms after evoking tetanic stimulation). Data for total muscle force (F_t) in relation to muscle-tendon complex length were fitted with a polynomial function using a least squares criterion

$$F_t = b_0 + b_1 x + b_2 x^2 + \cdots + b_n x^n \quad (1)$$

where x represents muscle-tendon complex length. b_0, $b_1 \ldots b_n$ are coefficients determined in the fitting process. Data for passive muscle force (F_p) in relation to muscle-tendon complex length were fitted with an exponential function using a least squares criterion

$$F_p = e^{a_1 + a_2 x} \quad (2)$$

where x represents passive muscle-tendon complex length and a_1 and a_2 are coefficients determined in the fitting process.

Polynomials that best described the experimental data were selected by using one-way analysis of variance (ANOVA) [30]: the lowest order of the polynomials that still added a significant improvement to the description of changes of muscle-tendon complex length and muscle force data were selected. These polynomials were used for two purposes (1) averaging of data and calculation of standard deviations. Per each muscle studied, muscle forces at different TA muscle-tendon complex lengths were obtained by using these functions. Per each TA muscle-tendon complex length, forces were averaged and standard deviations (SD) were calculated to determine the muscle's force (mean ± SD). (2) Determining the maximal TA force and the corresponding muscle length. For each individual TA muscle, the maximal TA force is defined as the maximum value of the fitted polynomial for total muscle force and the corresponding TA length is determined.

One-way ANOVA was also used to test for the effects of BTX injection on the TA muscle's length range of force production, i.e., the range between muscle active slack length and the length at which maximal force is measured. Two-way ANOVA for repeated measures (factors: TA muscle-tendon complex length and animal group) was performed separately for the forces of each muscle. Differences were considered significant at $p < 0.05$. If significant main effects were found, Bonferroni post hoc tests were performed to further locate significant force differences within the factors [30].

Spearman's Rank correlation coefficient was calculated to test if reductions in TA total forces due to BTX-A injection are correlated with TA muscle-tendon complex length. Reduction in force is calculated as the difference in mean force between the control and the BTX animal groups at each TA muscle-tendon complex length and expressed as a percentage of the mean force of the control group. Correlations were considered significant at $p < 0.05$.

Results

Figure 2 shows superimposed examples of force–time traces for muscles of the anterior crural compartment at 5 sample TA lengths selected from the range of 13 TA lengths tested between muscle active slack length and the length 2 mm over the length at which the highest TA force was measured.

TA Force-Length Characteristics. ANOVA (factors: TA length and animal group) showed significant main effects on TA total forces, as well as a significant interaction. Post hoc test located significant major effects of BTX-A injection at most muscle lengths ($\Delta l_{mt\ TA} \geq 4$ mm). A significant negative correlation was found between reductions in TA total force with increasing TA length. Spearman's rank correlation coefficient was −0.94 ($p = 0.0049$). BTX-A caused TA total force to decrease by, e.g., 55.9% at $\Delta l_{mt\ TA} = 4$ mm, by 47.3% at $\Delta l_{mt\ TA} = 10$ mm (i.e., the length at which the maximum TA force was measured) and by 46.6% at the highest muscle length studied (i.e., $\Delta l_{mt\ TA} = 12$ mm). The length range of force production for the BTX group (9.46 ± 1.45 mm, mean ± SD) was not significantly different from that of the control group (10.35 ± 1.42 mm, mean ± SD).

ANOVA showed significant main effects also on TA passive forces, as well as a significant interaction. Post hoc test showed

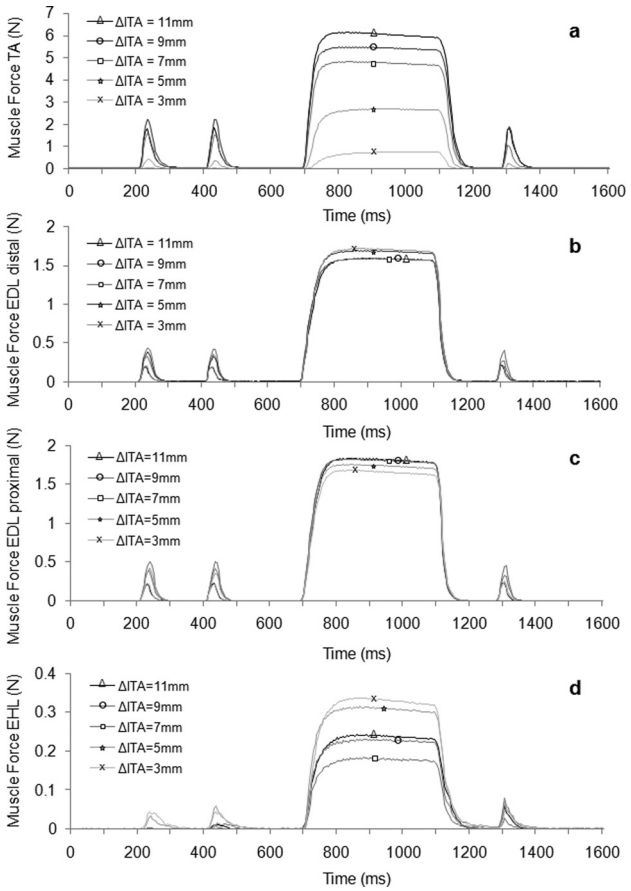

Fig. 2 Typical examples of force time traces measured at tendons of muscles of the anterior crural compartment. Superimposed traces recorded at 5 of the 13 total TA muscle lengths studied (*a*) the TA force, (*b*) the distal EDL force, (*c*) the proximal EDL force, and (*d*) the EHL force.

significant effects of BTX-A injection at higher lengths ($\Delta l_{mt\ TA} \geq 10$ mm): passive forces were higher for the BTX group (maximally by 43.9%) (Fig. 3).

EDL Forces. Both distally and proximally, ANOVA showed only a significant effect of BTX-A injection on EDL total forces; but no significant effects of TA length or a significant interaction. The mean force decreases BTX-A caused for the TA lengths studied were 67.8% distally and 62.9% proximally (Figs. 4(*a*) and 4(*b*)). In contrast, ANOVA showed only a significant effect of TA length on EDL passive forces; thus neither significant effects of BTX-A injection nor a significant interaction.

ANOVA also showed significant main effects on the EDL proximo-distal total force differences (Fig. 4(*c*)) and a significant interaction. For the control group, the EDL distal forces were higher than proximal forces for $\Delta l_{mt\ TA} < 5$ mm and vice versa at higher TA lengths. Increasing the TA length was shown to change the force difference measured at $\Delta l_{mt\ TA} = 0$ mm significantly for $\Delta l_{mt\ TA} > 6$ mm. For the BTX group, the EDL proximal forces were higher than the distal forces for all TA lengths; however, no significant effect of increasing TA length was shown. Post hoc test located significant effects of the BTX injection on the EDL proximo-distal total force differences for $\Delta l_{mt\ TA} < 5$ mm.

EHL Forces. ANOVA showed significant main effects on EHL total forces, but no significant interaction. The mean force decrease BTX-A caused for the TA lengths studied was 9.2% (Fig. 5). For both animal groups, the increased TA length caused the EHL forces to decrease significantly (by 34%) within almost the entire length range ($\Delta l_{mt\ TA} > 2$ mm) compared to EHL force measured at $\Delta l_{mt\ TA} = 0$ mm (post hoc). Regarding EHL passive forces, ANOVA showed neither significant main effects nor a significant interaction.

In summary, the present results make it evident that BTX-A administration causes the forces of not only the injected but also the noninjected muscles of an entire intact compartment to decrease. This confirms our first hypothesis. The results did show the existence of a significant correlation between injected muscle's length and the effects of BTX-A such that the force reductions decrease as the length of the muscle increases. The results also support the second hypothesis and show that BTX-A exposure has effects on the EMFT mechanism.

Discussion

Effects of BTX-A on the Forces of the Injected and the Noninjected Muscles of the Compartment. An important finding is that force decreases caused by BTX-A are muscle length dependent such that the increased length of the injected muscle and the reduction in force are negatively correlated. Similar results were shown in the study of Yaraskavitch et al. [14] for the cat soleus muscle and were inferred from their torque-joint angle data by Longino et al. [13] after testing the quadriceps musculature of the rabbit. These findings obtained from different muscles suggest that muscle length dependency of the effects of BTX-A are important to consider. This may have clinical implications some of which are discussed in a successive paragraph.

Yaraskavitch et al. [14] based their explanation for the observed change in the force-length curve, i.e., a less steep ascending limb, on adaptation in the number of in-series sarcomeres, which could occur 4 weeks postinjection. However, in the present study, only 5 days postinjection, the occurrence of such adaptation is less likely. Denervation of adult mice muscle was shown to have no effect on the sarcomere number [31]. Therefore, the present results suggest that length dependent effects of BTX-A may not be due exclusively to changes in the number of in-series sarcomeres within the muscle. Instead, a mechanism that involves changes in lengths of in series sarcomeres may be responsible. For such mechanism, the fact that BTX-A causes paralysis of muscle fibers within parts of the muscle belly [7] is central. Due to that, BTX-A injection may lead to differences in two types of mechanical interactions occurring among the structures comprising the muscle-tendon complex (1) *muscle-tendon interactions:* tendon tissue has nonlinear force–deformation characteristics [22], and under lower magnitudes of forces, it has been shown to be more compliant [32,33]. For the BTX-A injected muscle, a general expected effect of the resulting reduction of muscle force is less extension of the tendon for the same muscle-tendon complex length. Therefore, a muscle-tendon complex length dependent shifting of the sarcomere lengths to higher lengths is plausible. (2) *Muscle fiber-extracellular matrix interactions:* muscle fibers and the extracellular matrix (ECM) are mechanically connected not only at the ends of the muscle fibers, but also along their full peripheral length [34–36]. Consequently, muscle fibers can interact with the ECM and hence with each other mechanically [37–40]. In tetanized muscle, this mechanism has been shown to limit shortening of muscle fibers after tenotomy [39] and aponeurotomy [41,42], although some muscle fibers lost their myotendinous connection to the insertion of the muscle, the ECM connected to the muscle fibers via trans-sarcolemmal molecules [34] prevents the sarcomeres within these muscle fibers from shortening to their active slack length. In BTX-A injected muscle due to a lack of stimulation, the paralyzed muscle fibers do not shorten as activated muscle fibers of a nonparalyzed muscle would. Therefore, the interaction mechanism described is expected to cause a resistance to the shortening of the sarcomeres within the activated muscle fibers.

A common indicated effect therefore appears to be the shifted lengths of the sarcomeres within the activated muscle fibers. Recently, Turkoglu et al. [43] studied the principles of effects of

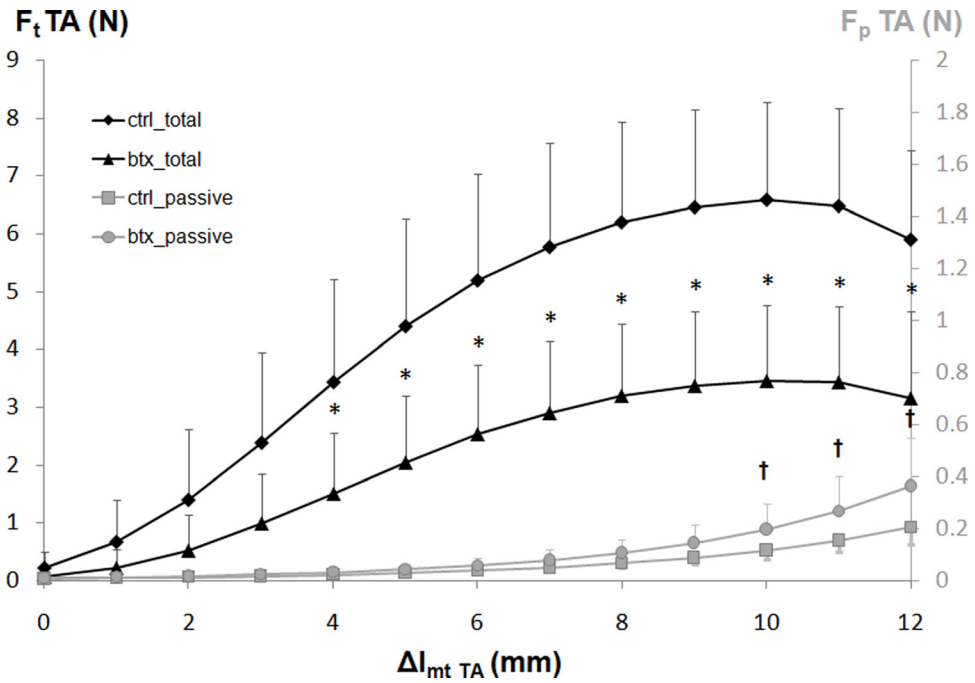

Fig. 3 The effects BTX injection to TA muscle on its isometric muscle force-length characteristics. Absolute total and passive isometric forces are shown as mean values ± SD for the control group and the BTX injected group of animals. The TA muscle-tendon complex length is expressed as a deviation ($\Delta l_{mt\ TA}$) from the active slack length of BTX injected group. Significant differences between the TA force of the BTX injected group and the control group (Bonferoni post hoc test) are indicated by * (total force) and by † (passive force).

BTX-A on muscular mechanics by extending a finite element model of rat muscle [44,45]. They activated only selected parts of the muscle, and the remainder parts were considered to represent paralyzed muscle fibers. The model results show in agreement with the arguments posed above that sarcomeres do attain higher lengths. Consequently, if the active sarcomeres of the partially paralyzed muscle are at the ascending limb of their force-length curve, they can produce more force compared to their counterparts in the non-paralyzed muscle. This suggests that a *net* muscle force reduction effect of BTX-A originates exclusively from the presence of paralyzed muscle fibers, as the active ones may have even an enhanced potential of active force production. Note that the model results [43] indicate that compared to lower muscle lengths, this potential compromising the effectiveness of BTX-A is greater at intermediate muscle lengths because there is relatively more resistance to sarcomere shortening. On the other hand, if the active sarcomeres are at the descending limb of their force-length curve, the opposite is valid, i.e., as they attain higher lengths, they produce less force. Note that in conditions similar to the present ones, i.e., for muscle with epimuscular connections to its surrounding structures, previous model studies suggest heterogeneity of sarcomere lengths within muscle fibers [23,28,46]. Particularly at higher muscle lengths, sarcomere length heterogeneity may include sarcomeres at both ascending and descending limbs of their force-length curves within the same muscle fibers [24,47]. Therefore, for such lengths, a more complex mechanism may determine the effectiveness of BTX-A in force reduction. Recall that the present results show a decrease in the force reduction effect of BTX-A at higher muscle lengths. According to the mechanism proposed, this suggests that most active sarcomeres may have been shifted to lengths favorable for force production. It is important to note that the mechanisms considered here to explain the length dependency of the effects of BTX-A are theoretical ones and they should be elaborated and verified in new studies. Medical imaging may be a feasible method to test these effects. Recently in no BTX-A injected condition has magnetic resonance imaging analyses shown the occurrence of local and heterogeneous muscle tissue deformations as caused by joint angle changes in human muscles *in vivo* [29]. Such deformations involve variable magnitudes of local lengthening occurring simultaneously with local shortening at other locations and indicate that distribution locally of lengths of muscle tissue is possible. Coupled with diffusion tensor imaging, such analyses may allow for quantification of length changes specifically along the muscle fiber direction [48].

BTX-A is used in treating patients with cerebral palsy, e.g., in the management of spastic equines gait [5,6] as well as in upper limb spasticity [49]. In these patients, spasticity is responsible for movement disorders and functional disability that can be characterized by a limited joint range of movement [50]. An improved understanding of how BTX-A affects the force production of muscle for different muscle lengths may be clinically relevant. However, the moment arm lengths of muscles vary with varying joint angles [51,52], which make it difficult to relate such understanding directly to joint movement. Therefore, based on the present results, it cannot be concluded that the effects of the treatment are variable for different joint angles. Muscle hypertonicity in spasticity [53–55] causes the joint to be forcefully kept in typically a flexed position in which the muscle is expected to be short. It may be important that a more pronounced muscle weakening effect is found for shorter muscle lengths. However, due to the indicated difficulty in relating muscle force-length properties to joint movement, it cannot be concluded that more pronounced effects are available for the joint positions that may correspond to short muscle lengths. Nevertheless, the findings of Longino et al. [13] may support this expectation because these authors showed that muscle weakness effects of BTX-A cumulatively on the rabbit knee extensors are knee joint angle dependent, and are more pronounced for more extended knee positions. More importantly, the results of the present study show that potentially all muscles within a compartment can determine how BTX-A administration affects the mechanics at the joint, even though only one of them is injected. A noteworthy implication of this finding is that the

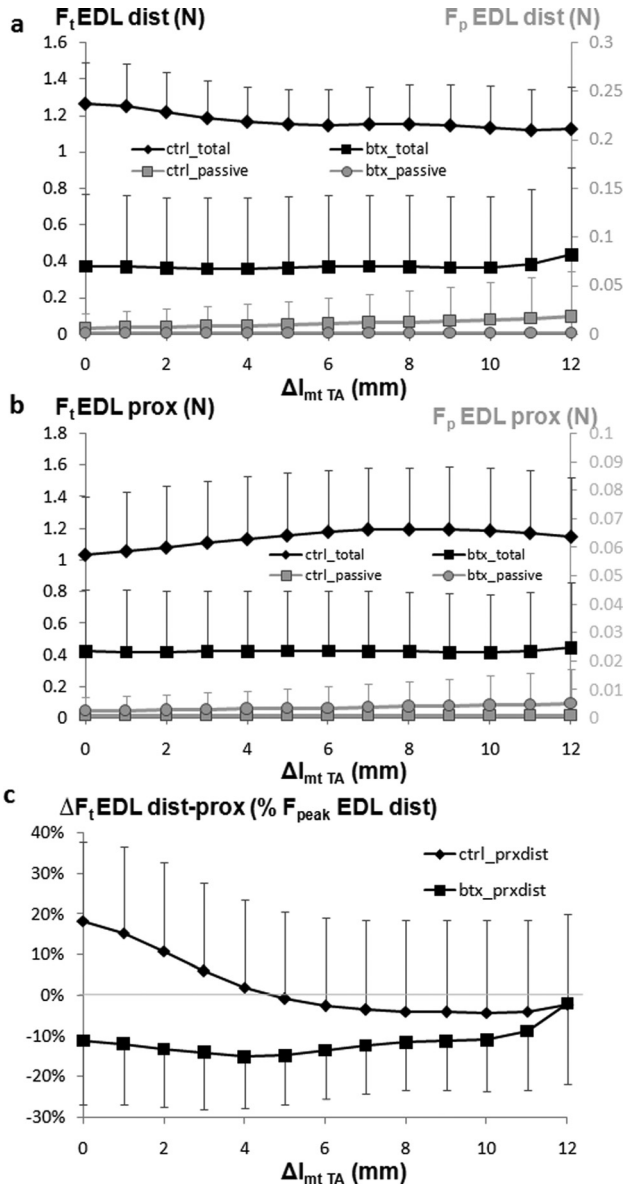

Fig. 4 The effects of BTX injection to TA muscle on the EDL forces as a function of increasing TA muscle length. (*a*) Absolute total and passive forces exerted at the distal EDL tendon. (*b*) Absolute total and passive forces exerted at the proximal EDL tendon. (*c*) Normalized proximo-distal EDL total force differences. The EDL forces measured from the control group and the BTX injected group of animals, plotted as a function of TA length, are shown as mean values ± SD. The TA muscle-tendon complex length is expressed as a deviation ($\Delta l_{mt\ TA}$) from the active slack length of the BTX injected group. Forces in (*c*) are normalized with respect to the EDL peak total distal force of the corresponding animal group (i.e., 1.27 ± 0.22 N and 0.44 ± 0.48 N, respectively for the control and the BTX injected group). Note that a positive force difference indicates that a *net* epimuscular myofascial load is exerted on the EDL in the proximal direction and a negative force difference indicates a distally directed *net* epimuscular myofascial load.

nontargeted muscles may have unintended effects also at the other joints that they span. These findings are expected to have clinical relevance and the experimental approaches developed are suitable for addressing them in new studies. These studies should also impose muscle length changes for the nontargeted muscles.

Injection Protocol Employed in Relation to the Effects on Muscle Forces. Cichon et al. [8] showed that the compound muscle action potential amplitude and the force exerted by lower hind limb flexors of the rat decreased predominantly in the first four days, with the decreases leveling off by the fifth day. Presently, the aim was to assess the short-term effects of BTX-A after stabilization. Therefore, all injections were performed five days prior to testing.

Note that the present BTX-A injection protocol differs from common clinical practice: (1) the injected dose (0.1 U i.e., approximately 0.32 U/kg) was less than that used in patients for lower limb muscles (3–6 U/kg), including children with cerebral palsy [56–58]. (2) A single injection was made to the mid-belly of the target muscle instead of using multiple injection points [1,15,56,59]. However, although they are not capable of showing whether an effective distribution of toxin is achieved, the present results strongly suggest that a considerable paralysis did occur within the target muscle. Shaari and Sanders showed that for the rat TA muscle, even a dose of 0.02U (a fifth of the dose used presently) injected to the midbelly causes approximately a fifth of the total cross-sectional area to be paralyzed only 24 h following the injection [7]. Shaari and Sanders also reported that an effective distribution of toxin is possible. Therefore, the quantity of BTX-A injected does not represent a low dose for the rat TA muscle as the difference with respect to the clinically used quantities would suggest and the present injection protocol was a suitable one for studying the effects of BTX-A on this muscle.

On the other hand, BTX-A is reported to be highly diffusive [15]. Although it binds with high affinity to local targets within the muscle, a larger volume, single injection may cause that site to be saturated and thus allows the spread of toxin to neighboring structures [56]. Therefore, the present injection protocol may have promoted toxin leakage to the adjacent EDL and EHL muscles. Yaraskavitch et al. explained their results with leakage of BTX-A into the noninjected muscle [14]; additionally, it is possible that partial paralyzation of these muscles is responsible for the presently measured force decreases. Note that the spread of BTX-A beyond the injection site is considered to be a side effect [16,56] and has been argued to occur not only after localized injections [14,15], but may be determined by the dose and concentration of the injection [56]. New studies are indicated to test for the role of different injection protocols beyond the injection site within an intact compartment.

BTX-A Has Effects on EMFT. After imposing length changes to a muscle, EMFT previously has been shown to cause changes in forces of restrained muscles [e.g., 23,60]. The present results showed similar effects for the control group: (1) The increased TA length caused significant changes in EDL proximo-distal force differences. Note that such force differences [24,47,61] are characteristic effects of EMFT [26] and represent the resultant of epimuscular myofascial loads acting on the EDL muscle. These forces originate from stretching epimuscular connections, which include direct collageneous connections between adjacent muscles as well as structures such as collagen-reinforced neurovascular tracts and compartmental boundaries. They also include forces generated within the sarcomeres of neighboring muscles that are transmitted onto the EDL muscle via these structures (for a detailed discussion, see [26]). Initially, the present EDL proximo-distal force differences were in favor of the distal force indicating that a resultant epimuscular myofascial load was acting on the muscle in the proximal direction. At the initial TA length, the length of the EDL muscle restrained at the reference position was conceivably higher causing interconnecting epimuscular connections between these muscles to be stretched as a source of such proximally directed loads. However, the effect was reversed at higher TA lengths. (2) The increased TA length also caused EHL force to change significantly. No such EMFT effect on EHL muscle has been reported since in the previous studies, forces of this muscle were measured together with the forces of TA muscle via their tied tendons [47,62]. However, EMFT between EHL and EDL muscles was shown previously to occur after imposing EHL

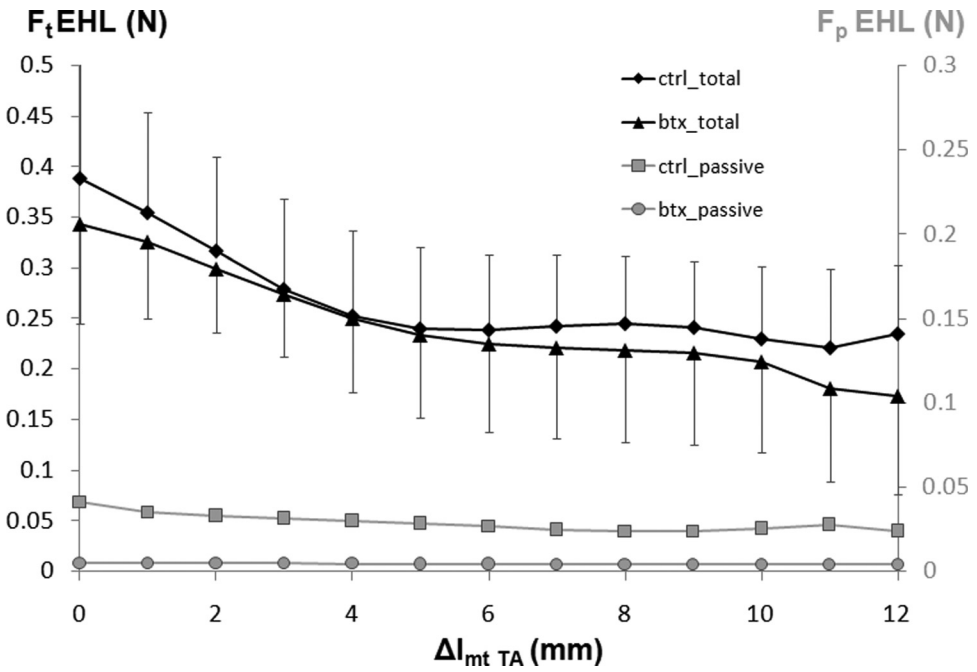

Fig. 5 The effects of BTX injection to TA muscle on the EHL forces as a function of increasing TA muscle length. Absolute total as well as passive forces exerted at the distal tendon of the EHL muscle measured from the control group and the BTX injected group of animals are shown as mean values ± SD. The TA muscle-tendon complex length is expressed as a deviation ($\Delta l_{mt\ TA}$) from the active slack length of BTX injected group.

length changes [23]. In those experiments, prior to testing, anterior crural compartment was opened and the TA muscle was removed. Therefore, only certain epimuscular connections of EHL muscle were left intact. In contrast, presently significant EMFT effects of TA length changes in a fully intact compartment were shown, which caused EHL forces to decrease approximately by a third.

The results showed that similarly to the control group, increased TA length caused significant changes in the EHL force such that the EMFT effects remained as profound in the BTX group. This finding can be interpreted as BTX-A did not affect EMFT between EHL and TA muscles. However, like all muscles within the compartment, a muscle weakening effect was found for the EHL muscle, indicating that its stiffness in the active state decreased. Yet, the length changes of the TA muscle yielded the same relative decrease in EHL force for both groups. Therefore, the results indicate two findings: (1) the epimuscular connections between EHL and TA muscles remained sufficiently stiff to allow the occurrence of EMFT and (2) BTX-A causes manipulation locally of their stiffness, operationalized for the changes of muscle relative positions imposed. This is in agreement with our expectation that BTX-A administration changes the interplay of stiffness of muscular tissues and their epimuscular connections, and confirms that BTX-A affected EMFT between the EHL and the TA muscles.

On the other hand, BTX exposure did affect EDL proximodistal force differences such that the effect of force differences initially in favor of the distal EDL force disappeared. This is an indicator that the epimuscular myofascial loads acting on the EDL muscle were manipulated by BTX. Also this result indicates that BTX-A administration changes the interplay of stiffness of muscular tissues and their epimuscular connections. Although it is not immediately apparent which component plays a dominant role, it is plausible that the epimuscular connections between the EDL and the TA muscles were less effective in EMFT after BTX-A administration.

A noteworthy effect presently shown is that at higher muscle lengths of the BTX-A injected TA muscle, the passive forces were significantly higher than those of the control group. A tenable explanation for this effect is an increased stiffness of the intramuscular connective tissues of the TA muscle and its epimuscular connections in combination. In agreement with this, the slope of the passive force-length curve of the BTX-A group was at least 86% higher than that of the control group for $\Delta l_{mt\ TA} \geq 10$ mm. Note that, in the passive state, any difference between the control and BTX groups in terms of existence of paralyzed muscle parts vanishes. Therefore, the increased passive TA forces cannot be ascribable to manipulated myofascial tissue stiffness, solely due to muscle relative position changes. Instead, it should also be considered that structural changes possibly occurred in these tissues. Within the first week following the denervation, atrophy of rat muscles was reported [63]. Also BTX-A was shown to cause atrophy [64,65]. Billante et al. showed that BTX-A causes decreased muscle fiber diameters as well as density [9]. Only for very high doses of BTX-A, even existence of fibrosis was observed by these authors within the muscle. However, no evidence is available whether passive muscle force increases accompanied such effects. Moreover, these effects are limited to the intramuscular tissues exclusively. The lack of direct data to show if tissue structural changes occurred presently is a limitation of this study. However, increased passive force of BTX-A injected muscle with intact epimuscular connections is an interesting finding, implications of which on tissue adaptation should be addressed in new specific studies.

The results show that BTX-A has effects on EMFT. However, these effects are not uniform within the anterior crural compartment for the conditions studied and they imply that EMFT is affected not only by muscle weakening but also by manipulated mechanical properties of the connective tissues comprising the EMFT pathways. EMFT has been regarded to play an important role in the mechanics of spastic paretic muscle [66] and surgical treatment techniques of the related functional deficiencies [19]. Such concepts are likely to have clinical implications also for the treatment of these conditions using BTX-A. New studies are indicated to explore further the relationship between the effects of BTX-A and those of EMFT.

Conclusions

The results show that exposure to BTX-A does affect the forces of all muscles operating in an intact compartment: (1) length dependent force decreases were found for the targeted TA muscle such that increased muscle length and the reduction in muscle force are negatively correlated. However, no change in the muscles' length range of active force production was found. (2) The simultaneously measured forces of the noninjected synergistic EDL and EHL muscles also decreased significantly, suggesting the presence of unintended additional effects both for the targeted distal joint and for the nontargeted proximal joint. The results also show that BTX-A exposure has effects on the EMFT mechanism. However, these effects are not uniform within the anterior crural compartment.

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