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UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Passive Mechanical Properties and Protein Composition of Skeletal Muscle
Change with Botulinum Neurotoxin A Treatment

A Thesis submitted in partial satisfaction of the Requirements for the degree Master of
Science

in

Bioengineering

by

Bryan Edward Thacker

Committee in charge:

      Professor Richard Lieber, Chair
      Professor Samuel Ward, Co-Chair
      Professor Robert Sah
      Professor Shyni Varghese

2008

The Thesis of Bryan Edward Thacker is approved, and it is acceptable in quality and form for publication on microfilm:

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Co-Chair

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Chair

University of California, San Diego

2008

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LIST OF ABBREVIATIONS

BTX – Botulinum neurotoxin treated

CONTRA – contralateral

pDAB – p-Dimethylaminobenzaldehyde

SDS-PAGE – sodium dodecyl sulfate – polyacrylamide gel electrophoresis

SDS-VAGE – sodium dodecyl sulfate – vertical agarose gel electrophoresis

SNAP-25 – synaptosomal associated protein of 25K

SNARE – soluble *N*-ethyl maleimide-sensitive factor, attachment receptors

TA – tibialis anterior

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ABSTRACT OF THE THESIS

The Passive Mechanical Properties and Protein Composition of Skeletal Muscle
Change with Botulinum Neurotoxin A Treatment

by

Bryan Edward Thacker

Master of Science in Bioengineering

University of California, San Diego, 2008

Professor Richard Lieber, Chair
Professor Samuel Ward, Co-Chair

The effects of Botulinum neurotoxin A on the passive mechanical properties of

skeletal muscle have not been researched but may have significant clinical effects in

the treatment of neuromuscular disorders including spasticity. Single fiber and fiber

bundle passive mechanical testing was performed on muscles treated with Botulinum

neurotoxin A. Myosin heavy chain and titin composition of single fibers was determined by gel electrophoresis. Muscle collagen content was determined using a hydroxyproline assay. Botulinum neurotoxin treated single fiber passive elastic modulus, stiffness, and slack sarcomere length was reduced from the contralateral side. Single fiber myosin heavy chain composition shifted from faster to slower isoforms after treatment. The average titin molecular weight in a fiber also increased after treatment. Fiber bundle passive elastic modulus and stiffness increased while collagen content per mass of muscle tissue increased 48 percent. The passive mechanical properties of muscle change after injection with Botulinum neurotoxin and may be clinically beneficial to patients with spastic muscle.

INTRODUCTION

The Botulinum neurotoxins are produced by *Clostridium botulinum* and are responsible for botulism. They have been characterized as the most potent known toxins. [Gill, 1982] These toxins target neuromuscular junctions, the link in the motor unit between the nervous system and the muscle, and prevent the release of acetylcholine through selective cleavage of synaptic proteins. [Blasi et al, 1993] Upon activation of a normal neuromuscular junction, the SNARE complex forms within the nerve terminus. [Hay, 2001] This complex is made up of a number of proteins including synaptobrevin, SNAP-25, and syntaxin. The SNARE complex transports acetylcholine-carrying vesicles to the pre-synaptic membrane and is required for the docking and fusion of these vesicles with the membrane. Subsequently, acetylcholine is released into the synaptic cleft. Acetylcholine then transverses the synaptic cleft and binds to acetylcholine receptors on the muscle cell, which initiates muscle contraction. Botulinum neurotoxin A specifically cleaves SNAP-25. [Blasi et al, 1993] Cleavage of this protein prevents the formation of the SNARE complex, after which acetylcholine-carrying vesicles no longer release their contents into the synaptic cleft and the signal for contraction is not transmitted from the nerve terminus to the muscle fiber. This results in paralysis of the muscle and has been described as chemodenervation.

While naturally occurring botulism is often fatal, small doses of the toxin have been used to effectively treat a variety of neuromuscular disorders. [Jankovic, 2004] These treatments are effective in cases involving over-stimulation of the muscle, which results in irregular movements, abnormal posture, discomfort, and pain. These

pathologies include dystonia in its various forms, blepharospasm, muscle spasm, muscle contraction headaches, as well as a variety of gastrointestinal and sphincter disorders. Botulinum neurotoxin is also used to treat muscle spasticity and contractures secondary to cerebral palsy, stroke, brain trauma, or multiple sclerosis. Spasticity is a velocity-dependent increased resistance to passive muscle stretch, which is at least partially explained by exaggerated muscle stretch reflexes. [Lee et al, 2002, Hornby et al, 2006] Benefits of successful Botulinum neurotoxin therapy in patients with spasticity include improved gait, [Hesse et al, 1994] decreased pain, [Wissel et al, 2000] and improved range of motion. [Love et al, 2001]

There are a number of biological and functional changes in response to Botulinum neurotoxin therapy. The effect of injection dose and volume on muscle force production has been determined in a rat model. [Hulst et al, 2008] In this study, rat tibialis anterior (TA) was injected with one, three, or six units per kilogram Botox® in a four, 20, or 100μL vehicle volume. One-month post injection, ankle dorsiflexion torque was reduced from normal in a dose dependant manner. Injection volume did not have a consistently significant effect on the dorsiflexion torque. Dodd, et al, demonstrated a shift in myosin heavy chain composition to slower isoforms in rat muscle following injection with various doses of Botulinum neurotoxin A. [Dodd et al, 2005] Another study found that, following treatment with Botulinum neurotoxin, the presynaptic nerve terminals at neuromuscular junctions form new sprouts which actively release acetylcholine as little as 4 days after treatment with Botulinum neurotoxin. [de Paiva et al, 1999] Within 4 weeks, post-synaptic acetylcholine

receptors also appear at the new endplates. During the second month post-treatment, normal synaptic activity slowly returns to the original endplates and the nerve sprouts begin to retract. Retraction of the sprouts is a process that requires more than three months. In clinical practice, chemodenervation from Botulinum neurotoxin injection is effective for two to three months, after which, the muscle must be reinjected to maintain the therapeutic effect. Repetitive injections in some patients lead to antibody formation and immunoresistance such that subsequent injections are no longer effective. [Jankovic & Schwartz, 1995]

Previous research has shown that single muscle fibers from spastic muscles have a passive elastic modulus nearly twice that of fibers obtained from normal patients. [Friden & Lieber, 2003] This change in single fiber intrinsic mechanical properties indicates a reorganization of intracellular load bearing proteins in response to spasticity. Since it is the major source of intracellular passive tension [Prado et al, 2005], a change in titin isoform, concentration, or organization would be the most likely cause of passive fiber elasticity modulation in spasticity. However, when titin isoforms in spastic muscle were determined by gel electrophoresis, there was no difference found in titin size or concentration between spastic and normal muscle. [Olsson et al, 2006] Extracellular collagen fibrils and intermediate filaments also contribute to passive mechanical properties and may be involved in the change in passive elasticity in spastic muscle fibers. Another study using spastic muscle fiber bundles was performed to determine the contribution of the extracellular matrix to the passive mechanical properties. [Lieber et al, 2003] This study showed that the

extracellular matrix of spastic muscle was hypertrophic and appeared to be severely compromised mechanically. The reason for decreased elastic modulus was not clear, although spastic extracellular matrix morphologically appeared disorganized.

Titin is a filamentous protein of megadalton size and is the third most abundant protein in skeletal muscle. [Labeit et al, 1997] It is oriented parallel to actin and myosin filaments and spans the half sarcomere. [Furst et al, 1988] The titin filament is elastic and provides for passive tension in both the muscle fiber [Bartoo et al, 1997] and, in conjunction with the extracellular matrix, the whole muscle. [Linke et al, 1994] Titin is bound tightly to myosin heavy chains and is inextensible within the A-band. In the I-band, however, titin is highly extensible under tension. [Itoh et al, 1988] In skeletal muscle, titin in the I-band is made up of immunoglobulin-like domains, the N2A region, and the PEVK region. These regions are differentially spliced in different muscles which gives rise to a spectrum of titin molecular weights and elasticities. [Freiburg et al, 2000] Some muscles express only one titin isoform while others may co-express two isoforms. [Prado et al, 2005] For example, cardiac muscle co-expresses two isoforms of titin, a more compliant N2BA isoform and a less compliant N2B isoform. Following ischemia-induced cardiomyopathy in a rat model, rat cardiac muscle shifts from almost exclusively N2B isoform to co-expression of N2B and N2BA. [Neagoe et al, 2002] The sarcomeric organization of dual isoforms is unknown although varying the relative composition of the dual titin isoforms may modulate passive elasticity on the single fiber level. [Neagoe et al, 2002]

The extracellular matrix also plays a role in the passive mechanical properties of bundles of fibers and the muscle as a whole. Skeletal muscle extracellular matrix forms a continuous structure throughout the muscle that provides structural support for individual muscle fibers and plays an important role in force transmission between fibers and the tendon. The major structural protein of the extracellular matrix is fibrous collagen. The mechanical properties of fibrous collagen are dependant upon collagen concentration, crosslinking extent, and fiber orientation. [Charulatha & Rajaram, 2003; Pidaparti & Burr, 1992] Of the many known types of collagen, types I, III, and IV are found predominantly in the muscle and vary in relative quantity among the epimysium, perimysium, and endomysium. [Duance et al, 1977] Collagen content in muscle can change in response to altered use patterns or pathologies. For example, collagen concentration doubled in rat gastrocnemius, soleus, and TA three weeks after denervation. [Savolainen et al, 1988] Another study demonstrated increased collagen content in vastus lateralis biopsies from spastic cerebral palsy patients. Increased collagen content has been correlated with a clinical assessment of muscle spasticity severity. [Booth et al, 2001]

While much research has been published describing clinical outcomes of Botulinum neurotoxin therapy, there is relatively little information in the literature describing the effect of the toxin on muscle tissue. It has been shown that spasticity results in increased passive elastic modulus of single muscle fibers and reduced passive elastic modulus of the muscle extracellular matrix. [Friden & Lieber, 2003; Lieber et al, 2003] Since Botulinum neurotoxin has been effectively used to alleviate

the symptoms of spasticity and has an effect on the active mechanical properties of skeletal muscle, we now report whether the toxin also has an effect on the passive mechanical properties of single fibers and fiber bundles. Finding a significant effect on fiber and bundle mechanical properties, we then report the effect of Botulinum neurotoxin treatment on the key proteins in single fiber and extracellular matrix passive load bearing, which are titin and collagen respectively.

METHODS

Animal Model. Untrained, mature, male Sprague-Dawley rats (n = 24,

Harlan, Indianapolis, IN, USA), with a mean weight $386 \pm 5g$ ($\pm$ S.E.M), were used in

this study. Rats were housed two per cage at 20-23ºC with a 12:12h dark-light cycle.

All procedures were approved by the University of California and the VA Medical

Center Committees on the Use of Animal Subjects in Research.

Each rat was anesthetized under isoflurane (2%, 2.0L/min) and injected with

Botulinum neurotoxin A (Botox®, Allergan, Irvine, CA) in the right TA. Each dose

was 6U/kg usually in a 100μL volume although two rats each received this dose in a 4

and 20μL volume. The TA midbelly was located by palpation and the dose was

administered by injection at two sites of the TA midbelly. The contralateral TA was

left untreated and served as a control. Animals were maintained for one month after

which both Botulinum neurotoxin injected (BTX) and contralateral (CONTRA) TAs

were harvested under anesthesia. Previous data from our laboratory has demonstrated

an 80 percent reduction in ankle dorsiflexion torque at one month post-injection in this

model. [Minamoto et al, 2007] Animals were then euthanized with an intracardiac

injection of pentobarbital sodium (0.5ml of 390 mg/ml solution).

Harvested muscles were stored in storage solution at -20°C for up to four

weeks. Muscles were stored as either whole muscle or smaller muscle strips. The

storage solution was composed of 50% v/v glycerol and (in millimoles per liter):

potassium propionate, 170; EGTA, 5; Na_2ATP, 21.2; $MgCl_2$, 5.3; imidazole, 10; azide,

1; glutathione, 2.5; and leupeptin, 0.05. This solution prevented freezing of the tissue during storage at -20ºC.

Single fiber and fiber bundle mechanics. The passive mechanical properties were determined for both single muscle fibers and fiber bundles as described previously. [Friden & Lieber, 2003; Lieber et al, 2003] Relaxing solution was used to limit actomyosin interactions. This solution was made up of (in millimoles per liter): imidazole, 59.4; K(MSA), 86; Ca(MSA)$_2$, 0.13; Mg(MSA)$_2$, 10.8; K$_3$EGTA, 5.5; KH$_2$PO$_4$, 1.0; leupeptin, 0.05; and Na$_2$ATP, 5.1. Whole muscle or muscle strips were bathed in chilled relaxing solution. All subsequent dissection and cell manipulation was performed under a stereomicroscope (Leica Microsystems MZ16). For single fiber testing, a single fiber segment 2.0 to 4.5mm in length was teased away from the muscle using microsurgical forceps. Fibers with visible defects such as tears or kinks were discarded. Each end of the fiber segment was secured to a wire (Figure 1). These wires were fixed to a force transducer (Aurora Scientific Inc., Ontario, Canada, Model Number 405A, Force Range 10.0mN) and a micromanipulator. Suture loops (10-0 nylon) were used on each wire to hold the fiber in place. A transparent panel at the bottom of the chamber allowed a laser (635nm) to pass through the chamber and the suspended fiber. The diffraction pattern produced by the sarcomere grating was collected by a photodiode array above the chamber. Sarcomere lengths were calculated from the distance between first or second order peaks in the diffraction pattern and the distance from the fiber to the photodiode array. The entire apparatus could be translated in the horizontal plane using micromanipulators on the stage.

After the fiber was mounted in the experimental chamber, the fiber was extended until the force transducer output was approximately 2μN. Fiber diameter was measured using cross hairs in the eyepiece and translating the stage with the micrometers. Three diameter measurements were taken from the top view of the fiber. Another three fiber diameters were measured from the side using a mirror inclined 45 degrees from horizontal and parallel with the fiber. An average fiber diameter and average cross sectional area were calculated from the six diameter measurements. The fiber length was measured from suture loop to suture loop in a similar manner.

Once the dimensions of the fiber were measured, slack sarcomere length was recorded and the fiber was extended in a stepwise fashion with a two-minute stress relaxation period between each extension. A LabVIEW (LabVIEW 8.2, National Instruments, 2007) program was developed that timed extension periods, calculated sarcomere lengths from the photodiode output, and recorded output from the force transducer. Sarcomere length and tension in the fiber were recorded at the end of each stress relaxation period. The fiber was manually extended 250μm at the end of each stress relaxation period using the stage-mounted micrometers.

Stress in the fiber was calculated by dividing the fiber tension by the average fiber cross sectional area. Strain at each point was calculated as the increase in sarcomere length from slack sarcomere length divided by the slack sarcomere length. An elastic modulus for each fiber was calculated as the slope of the stress-strain curve. The stiffness of each fiber was calculated as the slope of the tension-sarcomere length curve. Since slack and failure sarcomere lengths were variable, modulus and stiffness

calculations were limited to sarcomere lengths between 2.5 and 4.0μm. This also limited mechanical testing to the descending limb of the sarcomere length/tension curve. Previous data from this laboratory shows that the working sarcomere length of the rat TA *in vivo* ranges from ~2.05 to ~2.50μm. [Peters et al, 2003] Therefore, this testing procedure determines passive mechanical properties at sarcomere lengths longer than those experienced by the muscle fibers during normal operation.

Fiber bundles were dissected by cutting clusters of intact fibers away from whole muscle tissue. Fiber bundles were tested in the same fashion as single fibers except that a three-minute stress relaxation period was used between bundle extensions. If the bundles tested were too large, the force transducer would reach its maximum output before the end of the test. For this reason, bundles were carefully prepared with average diameters at or less than 300μm. Bundles from CONTRA muscles contained approximately 7 fibers per bundle. Since BTX fibers have smaller cross-sectional area, bundles of the same diameter from BTX muscle contained approximately 17 fibers.

After mechanical testing, fibers and bundles were transferred to microfuge tubes and suspended in 10 and 50μL, respectively, of sodium dodecyl sulfate-vertical agarose gel electrophoresis (SDS-VAGE) sample buffer. The samples were stored at -80°C until analyzed by gel electrophoresis. SDS-VAGE sample buffer was comprised of 8M urea, 2M thiourea, 3% SDS w/v, 75mM DTT, 0.03% bromophenol blue, and 0.05M Tris-Cl, pH 6.8. [Warren et al, 2003]

Myosin heavy chain gel electrophoresis. The myosin heavy chain composition of single fibers was determined using a sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) technique. [Talmadge & Roy, 1993] The resolving gel was composed of 8% acrylamide/bis (50:1), 30% glycerol, 200mM Tris special, 100mM glycine, 0.4% SDS, 0.10% ammonium persulfate, and 0.05% TEMED and was overlaid with a stacking gel composed of 4% acrylamide/bis (50:1), 30% glycerol, 70mM Tris special, 4mM EDTA, 0.4% SDS, 0.10% ammonium persulfate, and 0.05% TEMED.

Standards for myosin heavy chain gels were prepared by homogenizing adult rat TA whole muscle. This standard contains each of the four adult skeletal muscle myosin isoforms. Single fiber samples were prepared by placing the sample tubes in 100ºC water bath for three minutes. Then, 1μL of CONTRA single fiber sample was diluted in 9μL of SDS-PAGE sample buffer and loaded onto the gel. Since BTX fibers were smaller in cross sectional area and contained less protein, 2μL of the BTX fiber sample was diluted in 8μL of SDS-PAGE sample buffer. The myosin heavy chain standard was loaded on every fourth lane. Gels were run at 4ºC for one hour at 10mA followed by 18-22 hours at 275V. Gels were fixed and stained following the Silver Stain Plus procedure (Bio-Rad, Hercules, CA). Using the standard lanes, bands were categorized by isoform type. The intensity of each band was quantified using a GS-800 Calibrated Densitometer (Bio-Rad) and the Quantity One 1-D Analysis software (Bio-Rad).

Titin gel electrophoresis. The molecular weight of titin in single fibers was determined using sodium dodecyl sulfate -vertical agarose gel electrophoresis (SDS-VAGE). This procedure has been described previously. [Warren, et al, 2003] An acrylamide plug was placed at the bottom of the gel to hold the agarose in place. The final composition of this plug was 12.8% acrylamide, 10% v/v glycerol, 0.5M Tris-Cl, 2.34% N,N'-diallyltartardiamide, 0.028% ammonium persulfate, and 0.152% TEMED. The composition of the agarose gel was 1% w/v Sea Kem Gold agarose (Lonza, Basel, Switzerland), 30% v/v glycerol, 50mM Tris-base, 0.384M glycine, and 0.1% w/v SDS. This solution was poured over the acrylamide plug and must be kept warm to prevent premature solidification.

Titin standards were obtained from human cadaver soleus and rat cardiac muscle, which have known molecular weights of ~3700kDa and 2992kDa, respectively. [Freiburg et al, 2000] These tissues were homogenized and stored at -80ºC until ready to be loaded on the gel. Before loading on the gel, these standards were diluted by placing 4µL human soleus standard and 8µL rat cardiac standard into 98µL sample buffer. This produces 110µL total which is enough standard for two gels.

Single fiber sample tubes were placed in boiling water for three minutes unless they had already been heated for myosin heavy chain analysis. After cooling, each CONTRA sample was diluted by adding 3µL sample to 7.2µL sample buffer. 0.8µL rat cardiac titin was also added to each sample. Since BTX fibers are generally smaller, more of the sample is needed to detect the protein on the gel. In most cases,

5µL BTX fiber sample was diluted with 4.2µL of sample buffer and 0.8µL of rat cardiac titin. These amounts were varied depending on the amount of protein found in each particular sample. Each well was loaded with 10µL of either standard or sample. Standards were loaded into every fourth well. Gels were run at 4ºC for 5 hours at 15mA constant current.

Agarose gels were fixed and stained according to the Silver Stain Plus procedure except that gels were dried for approximately 20 hours at 40ºC immediately following fixing. The gels were subsequently rinsed and stained as described in the Silver Stain Plus procedure. Relative mobility and intensity of each band was quantified using a GS-800 Calibrated Densitometer (Bio-Rad) and Quantity One 1-D Analysis software (Bio-Rad).

The relative mobilities of proteins on the gel were linearly related to the log of their molecular weights. The average correlation coefficient for the gel was determined from the three standard lanes containing human soleus titin and rat cardiac titin. Relative mobilities of the unknown rat TA titins were then based on the distance of those bands from the rat cardiac titin in each lane. The molecular weight of the unknown band was calculated from the relative mobility and the correlation coefficient. For fibers containing dual titin isoforms, an average molecular weight was calculated by weighting the contribution of each band by its relative intensity as determined by densitometry.

Hydroxyproline Assay. A hydroxyproline assay was performed to estimate the collagen content of BTX and CONTRA muscles. [Edwards & O'Brien, 1980]

About 100mg of tissue was dissected from whole muscle and hydrolyzed in 6N HCl at 100ºC for 18 hours. Samples were then neutralized to about pH 5 using NaOH and diluted 15x to less than 1µg/mL hydroxyproline. 0.5mL of Chloramine-T solution was added to 1mL of the diluted sample. Chloramine-T solution consisted of 1.41g Chloramine-T in 80mL of pH 6 buffer and 5mL isopropanol. The buffer consisted of 17g NaOH, 25g citric acid monohydrate, 60g sodium acetate trihydrate, 6mL glacial acetic acid, and 150mL isopropanol in 600mL distilled water. The acidity of the buffer was corrected to pH 6 with HCl. After addition of the Chloramine-T solution, 0.5mL p-Dimethylaminobenzaldehyde (pDAB) solution was added to the sample. This solution consisted of 15g pDAB in 60mL isopropanol and 26mL 60% perchloric acid. After incubation at 60ºC for 30 minutes, the concentration of hydroxyproline in the samples was determined by spectrophotometry at 550nm. Hydroxyproline standards were created and treated with Chloramine-T and pDAB like the samples and provided a standard curve for spectrophotometry. Hydroxyproline concentration was normalized to the mass of the original tissue sample.

Statistical Analysis. Dependent variables of fiber diameter, slack sarcomere length, modulus, stiffness, myosin heavy chain composition, and titin composition were screened for normality and homogeneity of variances. Mean values were compared between BTX and CONTRA groups by one-way analysis of variance and post-hoc Tukey tests. Linear regressions were performed to determine correlations between measured parameters. Significance was set at $p < 0.05$. Since dependent

variable variance among single fibers was greater than variance among animals, each

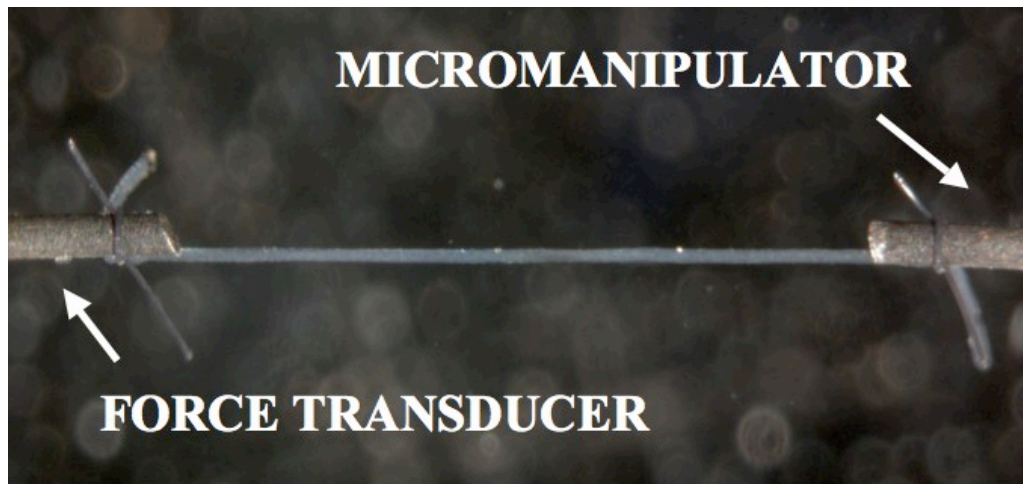single fiber and bundle was treated as an individual sample.

Figure 1: Single Fiber Mechanical Testing Setup. Single fibers were tied between a force transducer and a micromanipulator to perform passive mechanical testing. Suture loops were used to secure the fiber to the needles. Bundles were tested in a similar manner.

RESULTS

During the one-month post-injection period, rat weight increased to an average of 424.1 ± 5.4g (± S.E.M.). After harvesting, the CONTRA TA had a significantly greater mean mass than the BTX TA (Table 1, p < 0.001).

Single Fiber Mechanics. Fiber diameter and slack sarcomere length were measured during single fiber mechanical testing. Stiffness and elastic modulus were calculated from the force and sarcomere length data. The diameter of single fibers from the BTX TA (n = 64) was significantly reduced from the CONTRA TA (n = 66, Table 1, p < 0.001). Since the smallest BTX fibers were too small to produce a detectable diffraction pattern, single fiber dissection on the BTX TA was biased toward larger fibers. Therefore, the mean diameter of fibers from the BTX TA was likely overestimated. The mean slack sarcomere lengths for BTX and CONTRA fibers were 2.194 ± 0.025μm and 2.295 ± 0.016μm, respectively (Graph 1, p < 0.005). The mean stiffness of CONTRA fibers was 0.258 ± 0.014N/μm and was nearly a three-fold increase over the mean stiffness of BTX fibers, which was 0.085 ± 0.005N/μm (Graph 2). Since stiffness is calculated as the slope of the passive tension/sarcomere length curve and is not normalized by cross sectional area, the difference between groups is largely based on difference in fiber size and reflects fiber extrinsic properties. Linear regression of fiber diameter and stiffness revealed a highly significant correlation for both BTX (Graph 3, R^2 = 0.368, p < 0.001) and CONTRA sides (R^2 = 0.577, p < 0.001). The elastic modulus is the slope of the stress/strain curve between sarcomere lengths 2.5 and 4.0μm. This measure is normalized for fiber

cross-sectional area and, therefore, indicates the intrinsic passive mechanical properties of the fiber. The average elastic modulus of CONTRA fibers was 63.434 ± 2.396kPa while the modulus of BTX fibers was 53.002 ± 2.631kPa (Graph 4, $p < 0.005$).

Fiber Bundle Mechanics. Mean bundle diameters were not significantly different between BTX and CONTRA legs (Table 1, $p = 0.726$). Mean bundle slack sarcomere lengths were 2.267 ± 0.060µm on the BTX leg and 2.253 ± 0.029µm on the CONTRA leg (Graph 1, $p = 0.865$). Mean bundle stiffness and modulus were both significantly different between BTX and CONTRA sides. Mean stiffness values for BTX injected and CONTRA bundles were 7.465 ± 0.807kPa and 4.170 ± 0.703kPa, respectively (Graph 5, $p < 0.005$). Since diameters were not significantly different between BTX and CONTRA bundles, the difference in stiffness represents a change in intrinsic mechanical properties. Mean modulus values for BTX and CONTRA bundles were 168.834 ± 29.599kPa and 75.140 ± 6.034kPa, respectively (Graph 4, $p < 0.01$). The modulus of CONTRA bundles is not significantly greater than the modulus of CONTRA single fibers ($p = 0.062$). Furthermore, the modulus of bundles was not correlated with the modulus of single fibers adjacent to the dissected bundle.

Myosin Heavy Chain Gel Electrophoresis. SDS-PAGE for myosin heavy chain composition was performed on 19 CONTRA and 24 BTX fibers (Figure 2). Of the CONTRA fibers, 12 expressed type IIB myosin only, 6 co-expressed types IIB and IIX, and one co-expressed types IIA and IIX. Among BTX fibers, all but two co-expressed myosin heavy chains type IIB and IIX. One fiber expressed only type IIB

myosin and the other co-expressed types IIA, IIX, and IIB. There were no significant differences in dependant variables based on fiber type. On average, myosin heavy chain in CONTRA fibers was composed of 71.98 ± 4.61 percent type IIB, 18.15 ± 1.92 percent IIX, and 9.23 ± 3.22 percent IIA (Graph 6). BTX fibers were composed of 56.85 ± 2.74 percent type IIB, 33.05 ± 2.09 percent type IIX, and 9.5 ± 1.1 percent type IIA. While myosin type IIA was unchanged by Botulinum neurotoxin injection, there was a significant shift in mean fiber composition from type IIB to type IIX indicating a slower average fiber type ($p < 0.01$).

 Titin Gel Electrophoresis. The titin composition of single fibers was also determined by gel electrophoresis (Figure 3). Of the 25 BTX single fibers tested for titin composition, 20 had only one band with a mean molecular weight of 3466.2 ± 8.4kDa (Graph 7). The remaining 5 BTX fibers had dual titin bands of mean molecular weights 3461.3 ± 9.8kDa and 3327.4 ± 17.9kDa, which were statistically different ($p < 0.001$). The heavier of the dual BTX titin bands was not significantly different than the single BTX band ($p = 0.959$). Of the 28 CONTRA fibers tested, 18 produced two distinct bands and the remaining 10 produced only one band. The mean molecular weight of titin from CONTRA fibers with an individual band was 3456.7 ± 19.1kDa and was not statistically different from the individual band of BTX fibers ($p = 0.601$). In CONTRA fibers with dual bands, the mean molecular weights of the upper and lower bands were 3423.9 ± 6.1kDa and 3307.8 ± 5.1kDa, respectively, and were statistically different from each other ($p < 0.001$). The heavier dual CONTRA band was almost significantly different than the single CONTRA band ($p = 0.054$).

An average molecular weight was determined for each fiber with dual titin bands. The contribution of either band to the average was determined by its relative intensity as measured by densitometry, thus creating a weighted average. Using this weighted average, the mean molecular weight of titin in all fibers from CONTRA TA was 3393.5 ± 12.8kDa (Graph 8). This molecular weight was significantly different from the mean molecular weight of fibers from BTX TA, which was 3454.4 ± 8.9kDa ($p < 0.001$).

Of all sarcomeric proteins, titin has been characterized as the greatest determinant of passive mechanical properties of the fiber and is proposed as responsible for setting slack sarcomere length and passive elastic modulus. [Wang et al, 1993; Bartoo et al, 1997] In this study, a significant correlation was found when the single fiber mean titin molecular weight was regressed with single fiber modulus (Graph 9, $R^2 = 0.127$, $p < 0.05$) but not slack sarcomere length (Graph 10, $R^2 = 0.026$, $p = 0.277$). These correlations were also examined when limited to only BTX or CONTRA limbs. Slack sarcomere length was correlated with the titin average molecular weight among the CONTRA fibers ($R^2 = 0.203$, $p < 0.05$) but not among the BTX fibers ($R^2 = 0.107$, $p = 0.128$). Fiber modulus was not significantly correlated with the titin average molecular weight among CONTRA fibers ($R^2 = 0.157$, $p = 0.055$) or BTX fibers ($R^2 = 0.234$, $p = 0.067$) although the square of the correlation coefficient improved when the analysis was limited to only one limb.

Hydroxyproline Assay. Total collagen content in whole tissue was determined on two CONTRA muscles and two BTX muscles. The average collagen

content (µg collagen per mg tissue) in CONTRA and BTX muscles was 0.69 and

1.02µg/mg respectively (Graph 11). Therefore, on average, BTX muscle has 48

percent more collagen per unit mass than CONTRA muscle. Since only four samples

were tested, no statistical analysis was performed on this data.

Graph 1: Slack Sarcomere Length. Mean slack sarcomere lengths in single fibers and fiber bundles. Error bars indicate S.E.M. Slack sarcomere lengths were statistically different between BTX and CONTRA sides for single fibers ($p < 0.005$) but not fiber bundles.
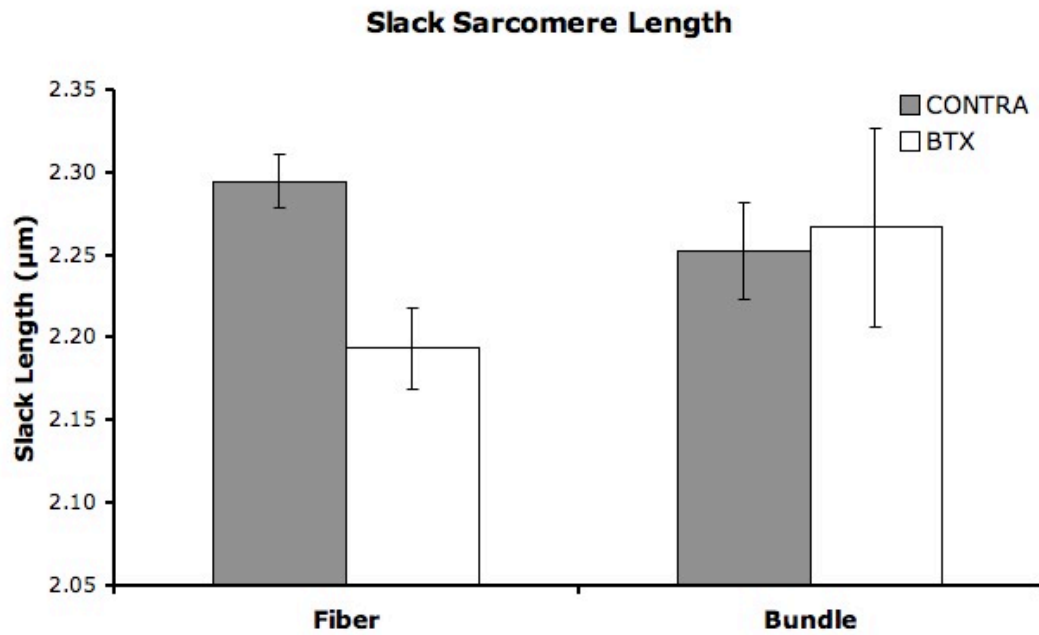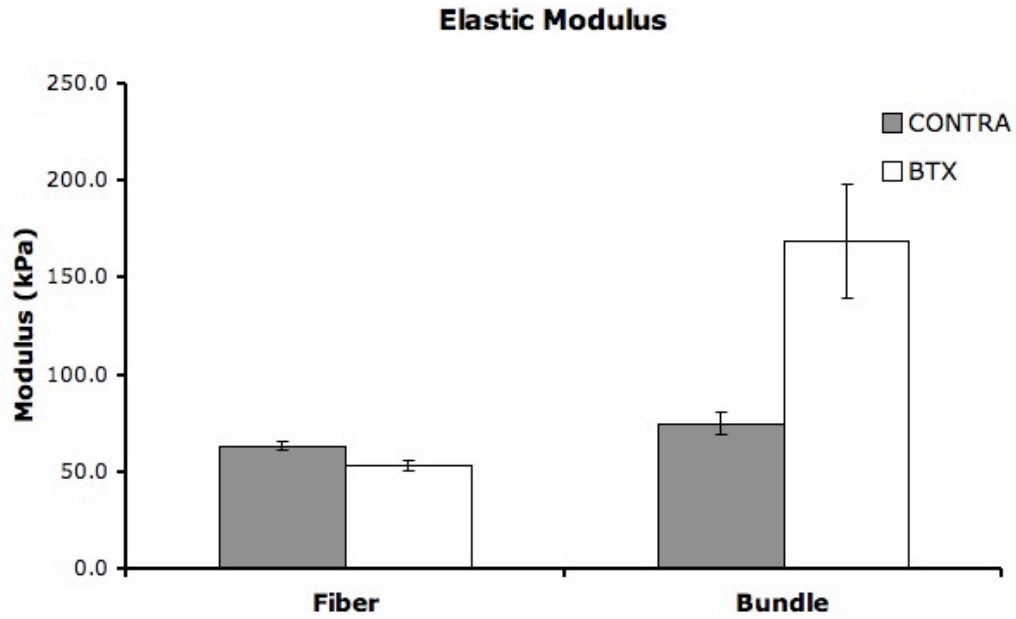
Graph 2: Fiber Stiffness. Error bars indicate S.E.M. Stiffness was significantly different between BTX and CONTRA sides (p < 0.001).


Graph 3: Fiber Diameter / Stiffness Correlation. Linear regression of fiber diameter and fiber stiffness. The correlation was highly significant for both BTX (R^2 = 0.368, p < 0.001) and CONTRA sides (R^2 = 0.577, p < 0.001).
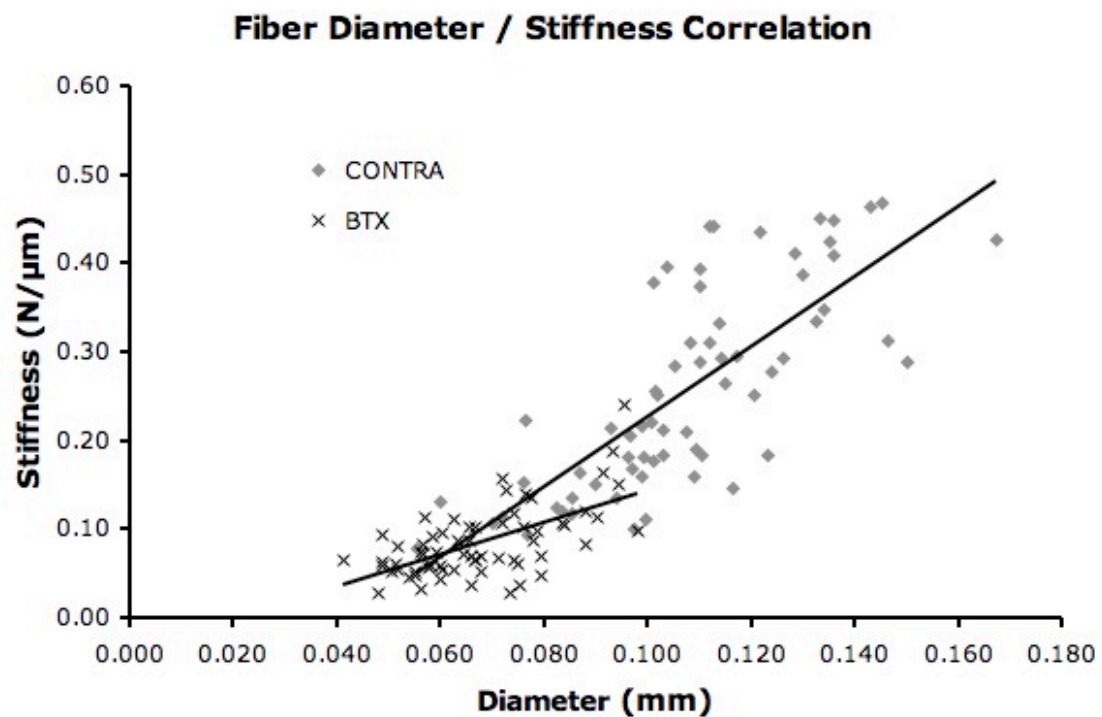
Graph 4: Elastic Modulus. Mean elastic modulus in single fibers and fiber bundles. Error bars indicate S.E.M. A significant difference was found between BTX and CONTRA sides for both single fibers ($p < 0.005$) and fiber bundles ($p < 0.01$).

Graph 5: Bundle Stiffness. Mean bundle stiffness. Error bars indicate S.E.M. Stiffness was significantly different between BTX and CONTRA sides ($p < 0.005$).


Bundle Stiffness
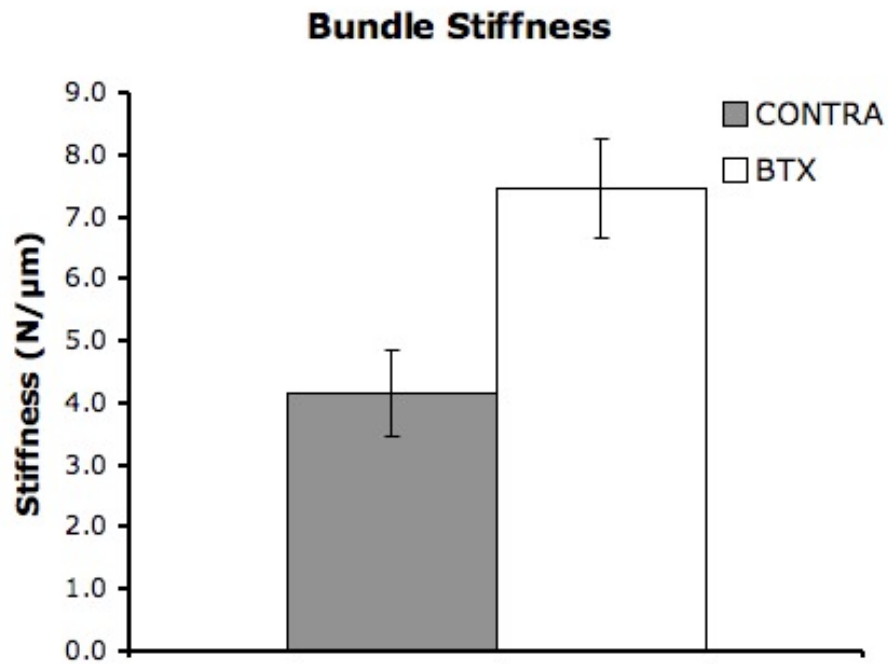
Graph 6: Fiber Myosin Heavy Chain Composition. Single fiber average myosin heavy chain composition. Error bars indicate S.E.M. A significant difference exists between BTX and CONTRA sides in type IIX and type IIB content ($p < 0.01$). Myosin heavy chain type I was rarely expressed in tested single fibers.

Fiber Myosin Heavy Chain Composition
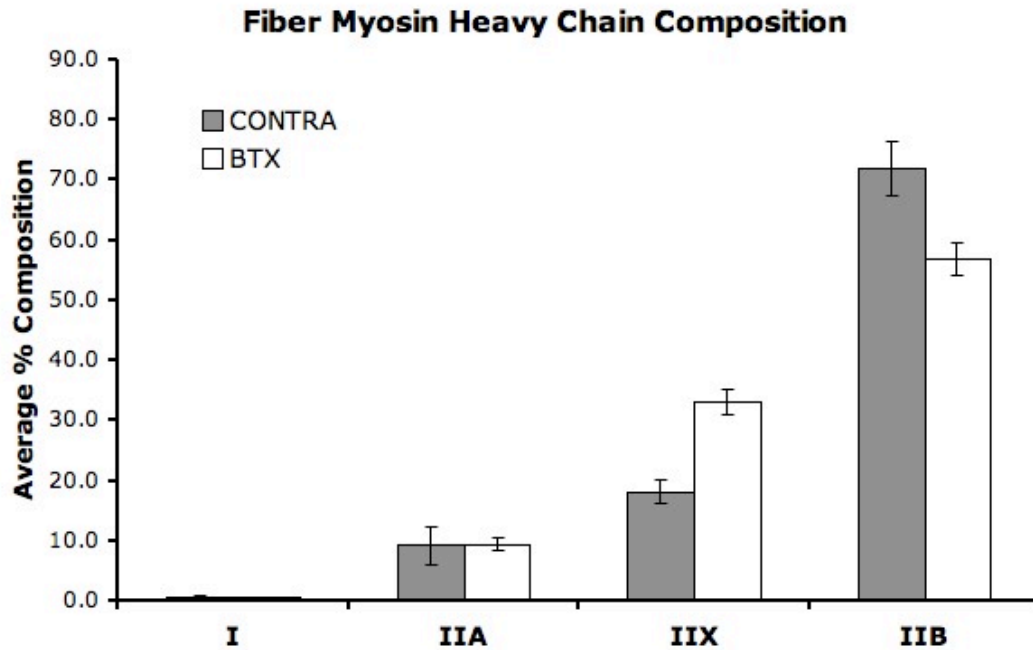
**Graph 7: Fiber Titin Composition.** Average molecular weights for titin from BTX and CONTRA single fibers. Error bars indicate S.E.M. For both BTX and CONTRA single fibers, the lower double band is significantly different from the higher double band and the single band (p < 0.01). The single band and the higher double band were not significantly different for BTX (p = 0.959) or CONTRA (p = 0.054) fibers.

Fiber Titin Composition
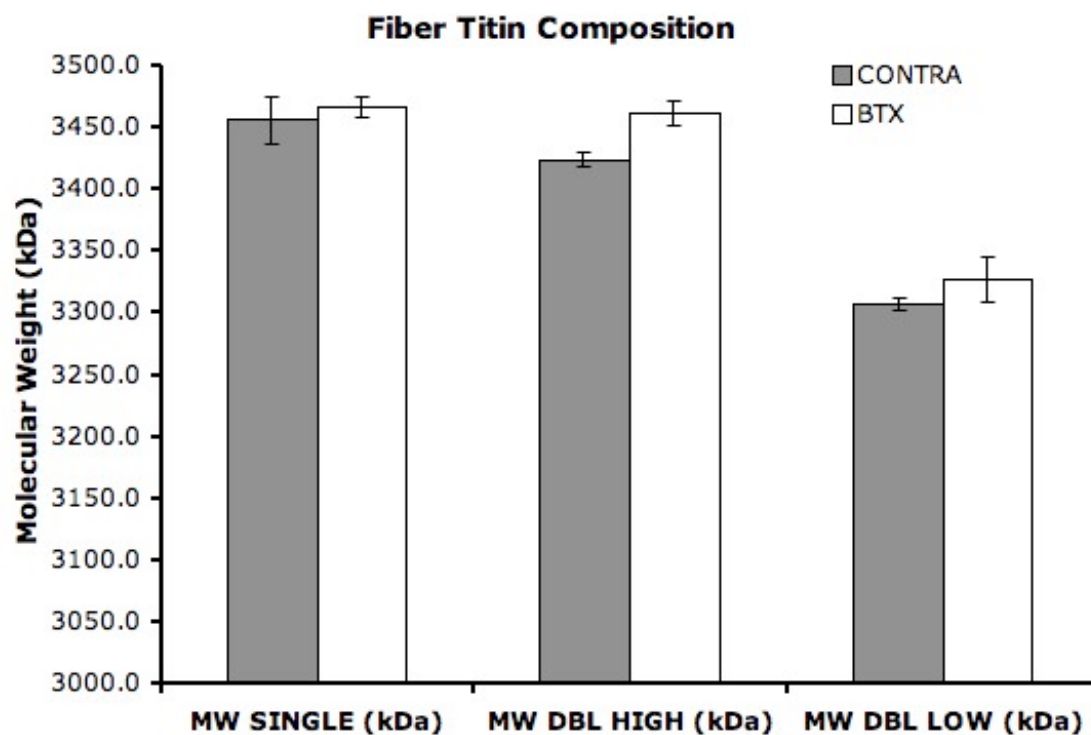
Graph 8: Fiber Titin Molecular Weight Average. Error bars indicate S.E.M. The contribution of each of the double bands was weighted by the relative intensity of the band on the gel. The average titin molecular weight is significantly different between BTX and CONTRA limbs (p < 0.001).
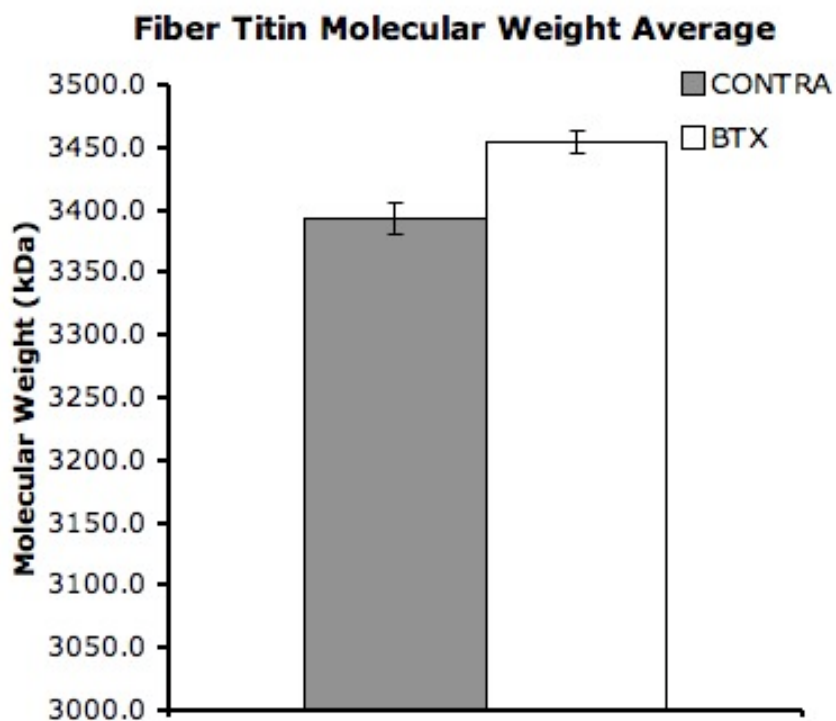
Graph 9: Fiber Titin Molecular Weight / Modulus Correlation. Linear regression of fiber average titin molecular weight and fiber modulus. This relationship is nearly significant for both BTX ($R^2 = 0.234$, $p = 0.067$) and CONTRA fibers ($R^2 = 0.157$, $p = 0.055$).
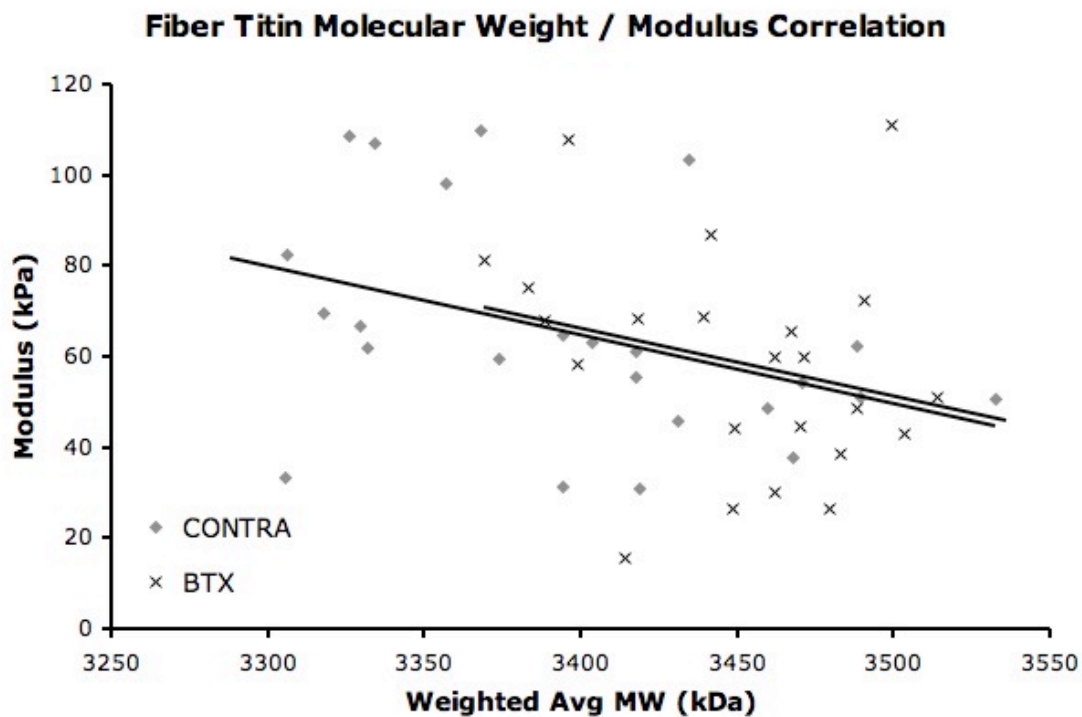
Graph 10: Fiber Titin Molecular Weight / Slack Sarcomere Length Correlation.
Linear regression of fiber average titin molecular weight and fiber slack sarcomere
length. This relationship is significant for CONTRA ($R^2 = 0.203$, $p < 0.05$) but not
BTX ($R^2 = 0.107$, $p = 0.128$) fibers.

Fiber Titin Molecular Weight / Slack Sarcomere Length Correlation
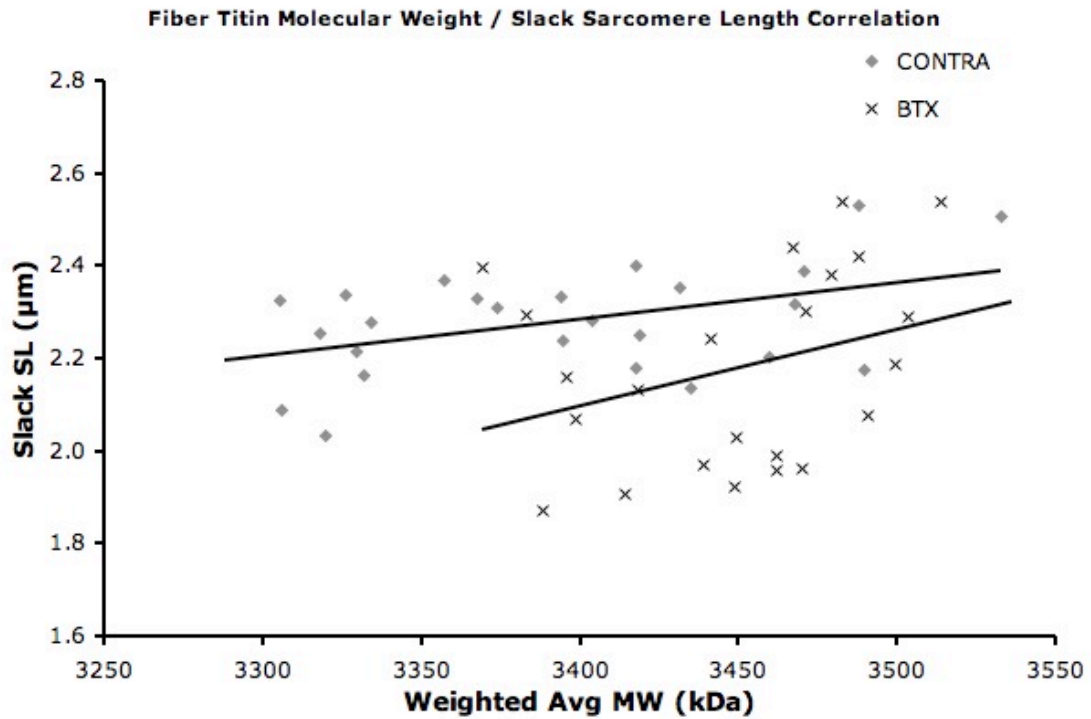
Graph 11: Muscle Collagen Content. Average collagen content in whole tissue. Collagen content in the BTX tissue was 48 percent higher than collagen content in the CONTRA tissue.

Muscle Collagen Content

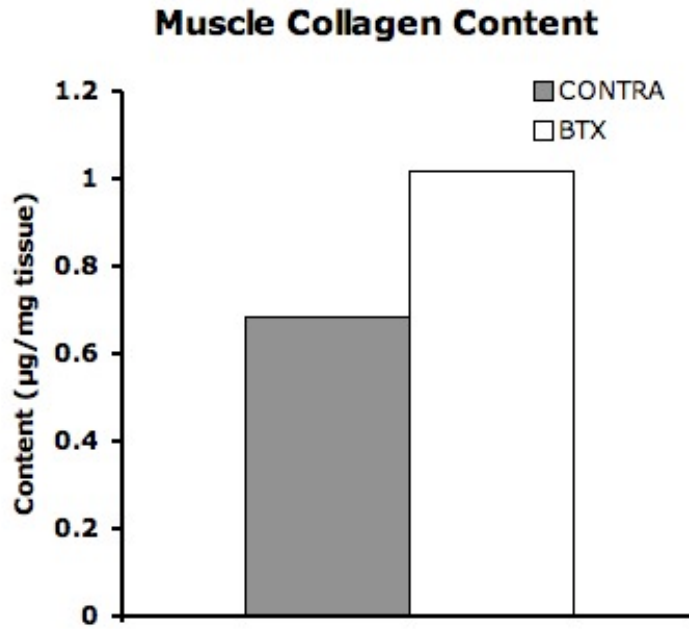**Table 1: Mean Values of TA Mass, Fiber Diameter, and Bundle Diameter.** (± S.E.M.) Statistical significance ($p < 0.001$) is denoted with an asterisk. TA mass and fiber diameter were significantly different between BTX and CONTRA sides.

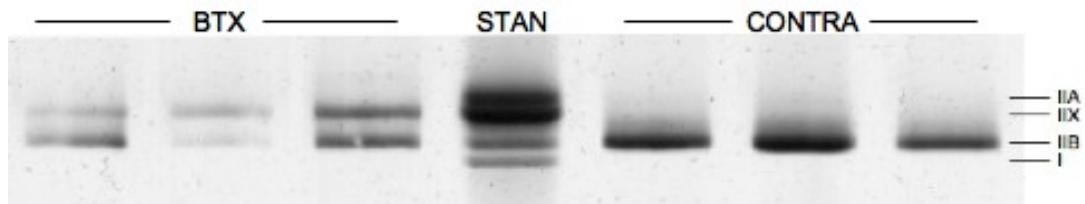|	TA Mass*	Fiber Diameter*	Bundle Diameter
CONTRA	0.892±0.014g	0.108±0.003mm	0.370±0.024mm
BTX	0.418±0.022g	0.067±0.002mm	0.357±0.027mm

Figure 2: SDS-PAGE for Myosin Heavy Chain Composition. Each lane contains protein from a single fiber except for the middle lane, which contains a standard made from whole TA homogenate. Most fibers are composed of one or two types of myosin heavy chain.
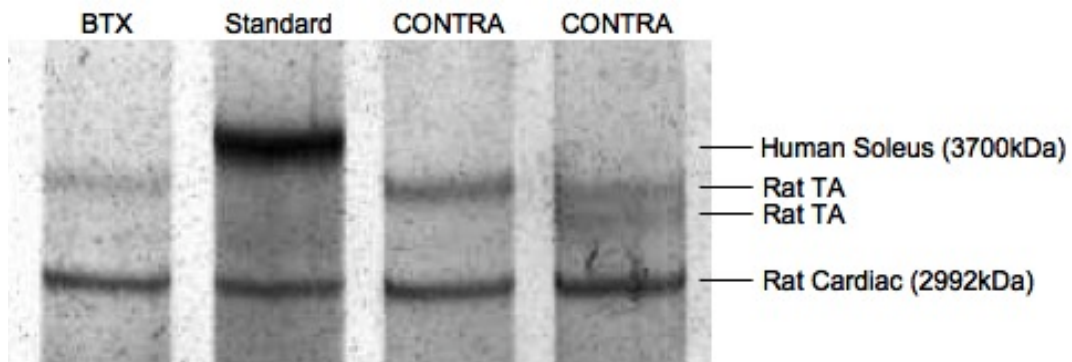
Figure 3: SDS-VAGE for Titin Composition. Each lane contains protein from a single fiber except the standard lane, which is loaded with homogenate from whole human soleus. Each lane is also loaded with rat cardiac homogenate. Fibers contain either one or two isoforms of titin that can be resolved on the gel. Relative mobility is linearly related to the log of molecular weight. Molecular weight is calculated for unknown single fibers using the human soleus and rat cardiac titin standards.

DISCUSSION

Passive mechanical properties and protein expression of the rat TA changed one month following treatment with Botulinum neurotoxin. TA mass and fiber diameter both decreased, as did single fiber passive stiffness, elastic modulus, and slack sarcomere length. The fiber bundle passive elastic modulus and stiffness increased after Botulinum neurotoxin treatment. These single fiber and fiber bundle changes in passive elastic modulus are opposite those observed in spastic muscles, while single fiber diameter and slack sarcomere length also decreased in spastic muscle. [Friden & Lieber, 2003; Lieber et al, 2003] These mechanical changes were accompanied by a shift in single fibers to a slower myosin heavy chain isoform and larger average titin molecular weight. Titin molecular weight, in some instances, was significantly correlated with passive elastic modulus and slack sarcomere length. Collagen content per tissue mass was increased in BTX muscle.

In a previous study, Botulinum neurotoxin has been shown to decrease TA mass and fiber diameter one month following treatment. [Minamoto et al, 2007] Decreased fiber diameter may have important functional implications for passive mechanical properties of the fiber. As surface area increases relative to fiber volume, passive load bearing structures at the surface of the fiber take on increased importance with respect to internal load bearing structures like titin. Passive load bearing structures at the fiber surface include the basement membrane and costameres. How these structures are affected by treatment with Botulinum neurotoxin is unknown.

Following treatment with Botulinum neurotoxin, the passive elastic modulus of single fibers was decreased 16.5 percent compared to the CONTRA side. This is a modest effect considering that the elastic modulus of spastic single fibers is nearly twice that of normal single fibers. [Friden & Lieber, 2003] An average spastic muscle fiber also has less than one-third the cross-sectional area of a normal fiber. Therefore, in spastic muscle, increased passive elastic modulus, an intrinsic property, is offset by decreased stiffness, an extrinsic property. Botulinum neurotoxin injection did, however, have a large effect on single fiber passive stiffness. Passive stiffness in BTX single fibers dropped 67 percent, on average, and was highly correlated with fiber diameter. As previously mentioned, selection of BTX fibers for mechanical testing was biased toward larger fibers, which likely results in an overestimate of the average passive stiffness. If this is true, BTX average fiber stiffness may actually be much less than is reported here. Thus, simply decreasing fiber size secondary to Botulinum neurotoxin treatment may have important clinical implications in moderating increased single fiber passive elastic modulus in spastic patients. While it has already been shown that spastic muscle fibers have significantly reduced single fiber cross-sectional area when compared to normal fibers, it is not known whether or not treatment with Botulinum neurotoxin further decreases the fiber diameter in spastic patients. If fiber diameter is reduced further, then passive stiffness of those fibers will likely also decrease. If fiber diameter does not decrease following treatment in spastic muscles, the change in passive elastic modulus as shown in this study will likely have a negligible effect.

Many fibers that were tested in this study were composed of two titin isoforms. Using two isoforms, a fiber may be able to modulate its myofibrillar elastic modulus by varying the relative amount of each isoform. For example, a fiber expressing equal amounts of each isoform may become more compliant by shifting the relative composition in favor of the larger isoform. In the most extreme situation, the titin composition in a fiber may shift completely to only one isoform. In this study, many fibers expressed only one titin isoform, yet still had a variety of passive elastic moduli. Other ways that the elastic modulus of a fiber could be adjusted with titin are varying the concentration of titin molecules in the fiber or changing the organization of titin filaments in the sarcomere.

Although titin has been characterized as the major intracellular determinant of passive elasticity, [Prado et al, 2005] the average titin molecular weight of a single fiber was not well correlated with the passive elastic modulus of the fiber. Among BTX fibers, average titin molecular weight accounts for only 23 percent of the variability in passive elastic modulus. The correlation in CONTRA fibers was not as strong, with titin molecular weight explaining only 15 percent of the variability in modulus. This weak correlation is similar to results in other studies demonstrating that the molecular weight of titin found in spastic muscle is no different than the molecular weight of titin found in normal muscle [Olsson et al, 2006] even though the passive elastic modulus of spastic muscle fibers is nearly twice that of normal fibers. [Friden & Lieber, 2003] In a separate study using fiber bundles from several different muscles types, the contribution of titin to the total passive load bearing of the bundle

ranged from 24 to 57 percent. [Prado et al, 2005] Other extra and intracellular passive load bearing structures accounted for as much at 39 percent of total passive tension in a bundle. [Prado et al, 2005] Thus, there is great diversity in the role of each passive load bearing structure amongst different muscles types. It is clear that other factors influence single fiber passive elastic modulus, which may include intracellular microtubules and intermediate filaments as well as extracellular collagen. [Granzier & Irvine, 1995]

The average slack sarcomere length in BTX fibers was 0.10μm shorter than the average slack sarcomere length in CONTRA fibers. Spastic muscle fibers also have reduced slack sarcomere length with respect to normal fibers although the magnitude of the change is about three times that seen after Botulinum neurotoxin treatment. While spasticity and Botulinum neurotoxin treatment both result in shorter slack sarcomere lengths, it is not known if slack sarcomere length shortening is compounded during treatment of spasticity with Botulinum neurotoxin. Single fiber average titin molecular weight was weakly correlated with slack sarcomere length among CONTRA fibers but not BTX fibers. Since slack sarcomere length is correlated with titin molecular weight in only CONTRA fibers, the contribution of titin to setting slack sarcomere length may be diminished in BTX fibers. Like passive elastic modulus, it also seems that structural components other than titin play a major role in setting single fiber slack sarcomere length. Other components, whose relative contributions to slack sarcomere length may have increased, must bear passive tension in the longitudinal direction of the fiber. Intermediate filaments run parallel to the

sarcomere and attach at Z-disks of the sarcomere. Also, the basement membrane, composed primarily of collagen, forms a sheath along the length fiber. Both of these components may play a role in setting slack sarcomere length and determining the passive elastic modulus of the fiber.

It is interesting that the slack sarcomere lengths in both CONTRA and BTX fiber bundles were not significantly different from each other and fell between the slack sarcomere length values for both CONTRA and BTX single fibers. It is possible that the extracellular matrix constrains these fibers to a uniform slack sarcomere length. When single fibers were dissected from the surrounding extracellular matrix for mechanical testing, the restraint may have been removed and fibers were free to assume the slack sarcomere length set by the intracellular or basement membrane components.

Myosin heavy chain composition in CONTRA fibers was mostly type IIB with relatively small amounts of type IIX and IIA. In BTX fibers, there was a moderate shift in composition from type IIB to the slower type IIX. This shift to slower average fiber type in response to Botulinum neurotoxin treatment has been demonstrated previously. [Dodd et al, 2005] Fiber myosin heavy chain composition was not significantly correlated with the passive mechanical properties or titin composition of either CONTRA or BTX fibers.

The mean passive elastic modulus of BTX bundles was more than twice the mean modulus of CONTRA bundles. Since the treatment effect on a single fiber passive elastic modulus was relatively small, a change of this magnitude in the bundle

suggests a change in the passive mechanical properties of extracellular structural material. The main structural component of the extracellular matrix is collagen. Collagen content per unit mass in whole BTX tissue was 48 percent higher than in CONTRA tissue and may account for the increased bundle passive elastic modulus. Collagen fibril orientation and extent of cross-linking also affect the mechanical properties of the extracellular matrix but these parameters were not tested in this study. [Charulatha & Rajaram, 2003; Pidaparti & Burr, 1992] Furthermore, it is unclear if increased collagen content per mass in BTX tissue results from increased collagen synthesis, decreased collagen degradation or is simply secondary to decreased muscle fiber mass in the tissue. In other studies, the rate of collagen synthesis has been determined by measuring the activity of two enzymes responsible for collagen biosynthesis, prolyl 4-hydroxylase and galactosylhydroxylysyl glucosyltransferase. [Savolainen et al, 1988] The activity of matrix metalloproteinases can also be determined to estimate the rate of collage degradation. [Somerville et al, 2003] As mentioned previously, smaller diameter fibers in BTX bundles increases the relative contribution of load bearing structures at the surface of the fiber relative to myofibrillar load bearing structures. These structures may play a role in increased passive elastic modulus of BTX fiber bundles but, in light of highly increased collagen content, this effect is probably small.

Fiber bundles taken from normal muscle have a passive elastic modulus more than four times the modulus of bundles taken from spastic muscle. [Lieber et al, 2003] Extracellular matrix in spastic bundles was also hypertrophic. A hydroxyproline assay

of muscle biopsy from patients with spasticity demonstrated increased collagen content when compared to normal muscle. [Booth et al, 2001] Taken together, these results suggest that the accumulated collagen in spastic muscle is not properly organized for passive load bearing like it is in normal muscle. Injection with Botulinum neurotoxin also results in increased collagen content and may partially reverse the mechanical deficiency found in spastic muscle if the additional collagen is properly oriented and crosslinked for passive load bearing. Whether or not well-organized collagen is accumulated in spastic muscle following Botulinum neurotoxin treatment remains to be tested.

The mass of fiber bundles was too small for the hydroxyproline assay and this assay was performed on whole tissue. Therefore, passive mechanical properties of fiber bundles could not be correlated directly to collagen content in the bundle. Furthermore, the hydroxyproline assay in this study was limited to two animals, which is not a sufficient sample size to draw definite conclusions. This study was also limited by the amount of protein found in single fibers. This amount was too small to accurately quantify after gel electrophoresis, as has been done in the literature. Finally, the conclusions drawn from these results in normal muscle are extended to the use of Botulinum neurotoxin in spastic muscle, which requires the assumption that normal and spastic muscle responds similarly to the toxin. Whether or not this assumption is true has not been tested.

Future work in this area should focus on the change in the quality and quantity of collagen in the extracellular matrix and the associated change in passive elastic

modulus of the fiber bundle. Passive mechanical testing could possible be performed on muscle strips of sufficient size for the hydroxyproline assay. Then, collagen content may be correlated with passive elastic modulus of the muscle strips. As previously stated, it is not clear if increased collagen content in BTX muscle is due to increased rates of synthesis, decreased rates of degradation, or decreased muscle fiber size. The activities of prolyl 4-hydroxylase, galactosylhydroxylysyl glucosyltransferase, and matrix metalloproteinases could be determined to differentiate between these three modes of collagen content change. Furthermore, changes in collagen content could be determined for the endomysium, perimysium, and epimysium as well as for specific collagen isoforms. The quality of collagen in spastic muscle and following Botulinum neurotoxin treatment could also be determined by quantifying collagen orientation and extent of cross-linking. Finally, changes in muscle secondary to Botulinum neurotoxin treatment could be tested in a model of muscle spasticity. Such a model has not yet been developed.

In conclusion, single fiber diameter and passive stiffness decrease after treatment with Botulinum neurotoxin. There were also small decreases in slack sarcomere length and passive elastic modulus. The passive elastic modulus of fiber bundles increased following treatment. These changes were paralleled by changes in titin molecular weight and collagen content. These data suggest that Botulinum neurotoxin treatment may mitigate the passive mechanical changes observed in spastic muscle.

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