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Recovery of Rat Muscle Size but Not Function More Than One-Year after a Single Botulinum Toxin Injection

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Abstract

Introduction—Neurotoxin injection is used to treat a wide variety of neuromuscular disorders. The purpose of this study was to measure the functional and structural properties of Botulinum toxin-injected adult rat skeletal muscle over nearly the entire rat lifespan.

Methods—Ten groups of animals were subjected to either neurotoxin injection (Botox[®], Type A, Allergan, Irvine, CA, USA; BT-A) or saline solution injection. Neurotoxin-injected animals (n=90) were analyzed at different time points: 1 week, 1 month, 3 months, 6 months, 12 months, or 18 months.

Results—In spite of the recovery of structural features such as muscle mass and fiber area by 12-months after neurotoxin injection, dorsiflexion torque production remained significantly depressed by 25% even at this time point.

Discussion—These data demonstrate that, after a single BT-A injection, while gross muscle morphology recovers over a 12-month time period, loss of contractile function does not.

Keywords

muscle physiology; muscle fiber type; neuromuscular junction; denervation neurotoxin; aging; Botox[®]

Introduction

Neurotoxin injection is commonly used to treat a variety of neuromuscular disorders. In light of the number and variety of neurotoxin injections that occur, as well as the economic impact of this procedure, it is of great interest to optimize injection efficiency, minimize side

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effects and understand the effects on muscle. Although rare^{1,2}, the side effects of generalized weakness, excessive loss of muscle tone, neurotoxin spread to adjacent regions and even death³ highlight the need to establish best practices for neurotoxin injection as well as to study the nature of botulinum neurotoxin type A (BT-A) effects on their target organ—skeletal muscle.

Since neurotoxin injection affects muscle mechanical function as well as its innervation, it is important to measure multiple structural and functional muscle properties when attempting to understand the effects of BT-A administration. For example, loss of voluntary or electrically induced muscle force could occur from decreased muscle fiber size, decreased number of innervated fibers or a combination of the two. In extreme cases, alterations in myosin heavy chain (MHC), the major muscle contractile protein, could also cause force alteration^{4,5}. The majority of studies to date measure only a few of these properties and assume a functional correlate (if structure is measured) or a structural correlate (if function is measured). In addition, since the effects of BT-A can be systemic⁶ it is important to differentiate the direct effects of toxin injection from those occurring systemically. Since we are also interested in the long-term effects of BT-A, a control group receiving only saline injection must also be employed to differentiate muscle performance changes due to BT-A injection from those that occur naturally with aging.

To address these concerns, we developed a highly accurate animal model in which the contractile properties of the rat dorsiflexor muscles can be measured with a coefficient of variation of only 10%⁷ and we analyzed dorsiflexion torque, and structural and biochemical properties of these muscles after a single toxin injection⁶. Given this low variability, effect sizes of ~10% can easily be detected. We have used this model to study the relationship between injection dose and volume⁸ on function, the effects of joint manipulation on efficacy and side effects⁶, the effect of multiple injections⁹ and even defined the complete transcriptional profile of muscle at various time points after injection^{10,11}. These previous studies pointed to an asynchronous response by muscle cells in which silencing of the neuromuscular junction (NMJ) secondary to cleavage of the SNAP-25 protein leads to rapid and transient transcriptional changes, but persistent functional changes. However, like others, our previous studies were performed at a single time point or primarily used a single method. To fully characterize muscle's response to BT-A, multiple measurements must be made over a reasonable time span at reasonable intervals. Therefore, the purpose of this study was to measure the functional properties of normal adult rat skeletal muscle over most of the rat's lifespan (18 months; about ¾ of the rat lifespan)¹² while also quantifying muscle fiber size, fiber type, collagen content and titin, to try to understand structural correlates to functional changes observed. This was performed to define the natural history of muscle's response to BT-A injection as well as to understand the underlying basis for changes in muscle function after a single, therapeutic BT-A dose.

Materials and Methods

Animal Subjects

Animals used in this study were untrained, mature male Sprague-Dawley rats (Harlan, Indianapolis, IN, USA) with an average size of 399±29 g (mean±S.D., n=90). Rats were

housed two per cage at 20–23°C with a 12h:12h dark:light cycle. All procedures were approved by the University of California and the V.A Medical Center Committees on the Use of Animal Subjects in Research. After terminal physiological experiments, animals were sacrificed with an intracardiac injection of pentobarbitol sodium (0.5 ml of 390 mg/mL solution).

Experimental Model

Animals were randomly divided into ten groups, and subjected to either BT-A injection (Botox[®], Type A, Allergan, Irvine, CA, USA) or saline solution injection. Neurotoxin injected animals were analyzed at different time points: 1 week (n= 8), 1 month (n= 19), 3 months (n= 8), 6 months (n= 8), 12 months (n=9), or 18 months (n=6). Five groups of saline-injected animals—1 week (n=4), 1 month (n=4), 3 months (n=4), 6 months (n=4), 12 months (n=10), or 18 months (n=6) served as controls for anesthesia, handling, aging and injection procedures. The investigators were not blinded to the experimental intervention or the identities of the experimental groups.

After anesthesia induction (2% isoflurane, 2.0 L/min), ankle isometric dorsiflexion torque was measured prior to injection, as previously described⁷. Briefly, dorsiflexors were activated via the common fibular nerve while torque was measured using a custom-designed dynamometer⁷. The dynamometer was created from a dual-mode servo motor (Aurora Model 305B, Aurora Scientific, Aurora, ON, Canada) which has a force resolution of 1 mN, a linearity of 99.8% and a step response time under 2 ms. The fibular nerve innervates the anterior compartment as well as the peroneus muscles. The peroneus longus and brevis produce a slight plantarflexion torque while the anterior compartment muscle (tibialis anterior, TA, extensor digitorum longus, EDL and extensor hallucis longus, EHL) produce a strong dorsiflexion torque. Based on the muscle architecture of the TA compared to the EHL and EDL¹³, the dorsiflexion torque produced is dominated by TA which produces ~85% of the torque⁷.

Three contractions were averaged to yield the value for maximal isometric torque, which has been shown to have a coefficient of variation of about 10%^{7,14} enabling reliable resolution of small changes in dorsiflexor function. After determination of initial torque, rats received either a one-time BT-A injection (dose 6.0 units/kg in a volume of 100 µL) or a 100µL volume of 0.9% NaCl solution into the midbelly of the TA muscle. This region was localized by palpating the largest bulk of the muscle, and the volume was administered by the same physician into two sites of the midbelly as previously described⁸.

At different time points (1 week, 1, 3, 6, 12, and 18 months), dorsiflexion torque was measured from both BT-A and saline-injected groups in both hindlimbs to measure systemic and aging effects.

Muscle fiber size analysis

Excised TA muscles were snap-frozen in isopentane cooled by liquid nitrogen (–159°C) and stored at –80°C for subsequent analysis. Muscle cross-sections (10 µm thick) were taken from the TA muscle midbelly. Sections were first treated with 1% BSA and normal goat and rat serum as blocking agents. Sections were incubated overnight with a polyclonal anti-

laminin antibody (Sigma, St Louis, MO; dilution 1:1000), and then with the secondary antibody, Alexa Fluor 594 goat anti-rabbit IgG (Invitrogen, Carlsbad, CA; dilution 1:200). The laminin antibody is used to label the fiber perimeter and facilitate fiber area quantification as previously described.⁶

Sections were imaged with a SPOT RT digital camera (Diagnostic Instruments, Sterling Heights, MI) on a Nikon Microphot SA epifluorescent microscope (Nikon, Tokyo, Japan) using a 10X objective with a G-2B filter set for red fluorescence. Muscle fiber size and filtering criteria were applied to ensure measurement of actual muscle fibers and not connective tissue pieces or artifacts as previously described⁶. Regions with circularity below 0.30 or above 1.0 were excluded to prevent inclusion of fibers that were obliquely sectioned which has the effect of artifactually increasing fiber area¹⁶

Myosin heavy chain analysis

Myosin heavy chain (MHC) isoform distributions were determined by adapting the gel electrophoresis technique previously described.¹⁷ Muscles were homogenized, and the myofibril-rich pellets were washed and resuspended in buffers supplemented with a protease inhibitor cocktail (100μM PMSF, 10μg/ml leupeptin, and 10μg/ml pepstatin A). Protein was then diluted in sample buffer to a concentration of 0.125 mg/ml across all muscle homogenates. Separation of MHC isoforms was performed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (16cm by 22cm, thickness: 0.75mm) with 22 h of migration at 275 V at 4°C. Stacking and resolving gels were 4% and 8% polyacrylamide, respectively. Gels were silver stained according to the manufacturer's instructions (Bio-Rad, Hercules, CA). The positions of MHC isoforms were determined by their relative electrophoretic mobilities, which have been extensively characterized previously^{18–20}.

Statistical analysis

Experimental results were analyzed by two-way analysis of variance with repeated measures using treatment group and testing time as grouping factors. *Post-hoc* Sidak tests were used to compare dependent variables among various pairs of groups. Relationship between muscle fiber area and torque was quantified by linear regression. All results are reported as mean ± SEM unless otherwise noted.

Previous publication of data subset

Three subsets of these data were reported as part of three previous studies^{9,7,11}. Details of the specific data sets are provided in the supplemental materials.

Results

Animals from both the BT-A and saline groups increased body mass through 12 months and then declined from 12 to 18 months due to the normal developmental and aging process (Fig. 1A). Body mass increased by about 50% throughout this study. While the only statistically significant difference in body mass between groups was observed at 18 months, animals in the BT-A group lost about 3% of their body mass on average over the first week.

Contralateral TA muscle mass remained relatively constant between 3 and 12 months, and declined between 12 and 18 months (Fig. 1B). In contrast, muscles injected with BT-A showed a significant decline in muscle mass after 1 month, began to recover at 3 and 6 months, but remained lower than the contralateral side until about 12 months (Fig. 1C). Since the mass of the TA is only about 1 g, changes in animal mass did not simply result from changes in TA mass.

Qualitatively, the morphology of BT-A injected TA muscles at different time points was as expected. In control muscles and muscles prior to injection, we observed tightly packed polygonal fibers with peripheral myonuclei and minimal mononuclear cells in the extracellular space. One week after injection, morphology was still fairly normal (Fig. 2A) but, after 1 month, the obvious change observed was decreased muscle fiber size with increased heterogeneity, as previously reported⁸ (Fig. 2B), which persisted at the 3-month time point (Fig. 2C). By 6 months, the tissue showed some regions of near normal fiber size and some regions of decreased fiber size, which were slightly hypercellular. Central nuclei were occasionally observed indicating some degree of fiber regeneration (Fig. 2D). Morphology of muscles at both 12 and 18 months (Fig. 2E and Fig. 2F) was indistinguishable from saline-injected muscles (not shown). Muscle fiber cross-sectional area (CSA) was correlated with muscle mass ($r^2=0.67$, $p<0.0001$) suggesting that a major fraction of the change in muscle mass was due to muscle fiber atrophy. Quantification of fiber area revealed that the largest decrease in fiber CSA was observed 1 month after injection, at which point it was only 28% of the value measured after saline injection (Fig. 3A). Fiber CSA gradually recovered to 63%, 74% and 96% of the contralateral values at 3, 6 and 12 months, respectively, and showed no difference compared to the saline injection group at either 12 or 18 months ($p>0.4$; Fig 3A). Fiber area on the contralateral muscles showed the same change as a function of age that was observed for muscle mass (Fig. 1B), namely, a gradual increase until about 12 months of age followed by a mild decline at 18 months of age.

Even though muscle mass and fiber size recovered after 12–18 months to those observed in saline-injected animals and contralateral controls, dorsiflexion torque production was significantly decreased at all time points from 1 week to 12 months when compared to the saline control groups (Fig. 4A). Negative dorsiflexion torque was observed at 1 week, presumably due to activation of peroneal muscles. Dorsiflexion torque gradually recovered to 45% and 50% of saline values at 3 and 6 months, respectively, and after 1 year, BT-A injected muscles generated ~73% of the torque generated by saline-injected muscles (Fig. 4A). The greatest variability in torque was observed for the 3-month experimental group, presumably due to variations in the rate of recovery from BT-A injection. Between 12 and 18 months of age, all groups decreased in torque generation, presumably due to age-related changes based on the fact that the decrease was observed in both the injected and contralateral limbs from the saline group (Fig. 4B). At the 18-month time point, BT-A and saline-injected muscles generated the same torque (Fig. 4A).

Several other biochemical assays revealed a minor response to BT-A injection. There was generally no change in titin mass as a function of time or between BT-A and saline-injected muscles (Supplementary Table 1), with the single exception being a very small increase in

titin mass for the BT-A-injected and their contralateral muscles at one month. Collagen content in the TA muscles was variable and somewhat irregular (Supplementary Table 2). Overall, collagen content increased in the BT-A injected muscles at the earlier time points. With regard to the contractile protein, MHC, high levels of type IIB MHC were expressed, followed by type IIX, IIA and type I (Supplementary Table 3). After BT-A injection, there was a significant increase in expression of the type I isoform at the expense of the fastest isoform (type IIB). Higher expression of type I muscle fiber was observed 1, 3, 6 and 18 months after BT-A injection when compared to saline groups ($p < 0.05$; Supplementary Table 3). However, while statistically significant, the magnitude of this effect was extremely small with type I MHC increasing from ~4% to ~7% of the total MHC pool.

Dorsiflexion torque correlated with TA fiber area as a function of time and treatment (Fig. 5; Supplementary Table 4). After 1 and 3 months there was a significant relationship ($r^2 > 0.6$) between the two parameters suggesting that the functional recovery was due to muscle fiber size recovery. After 6 months, there was no longer a correlation between the two parameters demonstrating that something other than muscle fiber size was responsible for the functional variability of these BT-A injected muscles.

Discussion

The results of this study reveal an asynchronous change in muscle fiber size, which did not track dorsiflexor functional properties completely. We observed a persistent loss in muscle contractile function even one year after a single BT-A injection, a time point at which all of the measured structural and biochemical parameters had returned to normal levels. These data, therefore, indicate that, after a single BT-A injection, while gross muscle morphology recovers over a 12-month time period, loss of contractile function persists.²³

The most rapid change observed was loss of dorsiflexion torque, presumably due to blockade of the NMJ, the mechanism of BT-A action²⁴. This blockage sets off a complex muscle transcriptional response involving ~2,000 genes¹¹ that leads to relatively rapid loss in muscle fiber size and mass over the first month⁶. This loss in muscle mass is due to upregulation of genes involved in destabilization of the NMJ (*Chrna1*, *Chrne*) as well as muscle genes involved in catabolic processes or atrophy (*Nfkb*, *Tgf- β* , *Fbxo32*). The net result of this transcriptional activity is rapid loss of muscle mass and, thus, at this one-month period, from a transcriptional point of view, the effect of BT-A has essentially been completed. Based on our previous study¹¹, there are only 32 transcripts that are expressed over control levels after the first month and these primarily relate to rebuilding lost muscle mass (*Myh12*, *Myh13*, *Tmod1*, *Ryr1*). These experiments indicate that NMJ blockade sets off a very rapid biological response that requires nearly the rest of the rat's lifetime to recover fully.

Since muscle structural and functional properties were measured from the same animal subjects, it was possible to apply correlation analysis to these structure-function data to gain insights into the relationship between the two. As expected, there was no significant correlation between fiber area and torque after 1 week. This is because the NMJ had been paralyzed rendering the muscle inactive, but fibers had not yet had sufficient time to atrophy.

This resulting asynchrony between biology, structure and function is most clearly seen by comparing muscle fiber area (structure) to dorsiflexion torque (function) across the time points measured. At the earliest time period measured after injection (1 week), torque decreased dramatically (Fig. 4A), even though muscle fiber area had not changed, presumably because the NMJ had been blocked (Fig. 3A). Then, muscle fiber size began to decrease in response to chemodenervation and torque remained low (1 month, Figs. 2B and 3A). Subsequently, during intermediate time periods (3–6 months, Figs. 2C, 2D and 3A) both muscle fiber area and dorsiflexion torque recover, albeit at different rates. Muscle fiber area recovered with an approximately 6-month half-life and was essentially at control levels one year after injection and indistinguishable from control muscle. Interestingly, torque was only about 75% of control values at this time point (Fig. 4A). Since muscle fiber size was near normal, and MHC isoform distribution were not dramatically altered (Supplementary Table 2), our interpretation of these results is that the muscle fibers were not normally functional even one year after the BT-A injection. The mechanistic site for this dysfunction could be the NMJ or the muscle fiber itself. From these data, we cannot distinguish between the two. However, if fibers are normally sized but function remains depressed, this indicates that, after a single BT-A injection, the initial chemodenervation sets off a rapid transcriptional response lasting weeks, muscle fiber size changes lasting many months, and neuromuscular reorganization that can require nearly the entire rat lifetime for full recovery. Evidence for such an irreversible denervation and reinnervation was provided in a recent study quantifying fiber type grouping after BT-A injection⁹. The mechanistic detail for such a response is not yet settled—whether the muscle fibers had the correct number and distribution of NMJs that simply did not function normally or whether the normal NMJ did not form. It has been stated that, after application of BT-A, the NMJ reforms in its original location with normal structure²⁵. However, physiological function of these NMJs with regard to quantal acetyl choline release, calcium currents, and other subcellular events has not been definitively tested²⁶. That fact that contralateral and saline-injected muscles showed similar structure and function suggest that the BT-A injection acted locally on the TA muscle, did not cause a significant systemic complication and that normal rat development occurred in spite of BT-A injection (Fig. 3B).

Analysis of the extracellular environment revealed a major increase in collagen concentration (Supplementary Table 2). While this was previously reported⁸, our interpretation of this measured collagen increase is that it actually over-represents the collagen response due to the selective loss of muscle fiber area (Fig. 3) and the fact that collagen is expressed per unit muscle mass. Because there is no quantitative model for the relationship between muscle fibers and extracellular collagen²⁷, it is not possible to convert collagen concentrations (μg collagen/mg tissue) into absolute numbers of collagen molecules per fiber. Based on the muted transcriptional response of extracellular matrix molecules¹¹ and the relatively large muscle fiber area change, we do not conclude that BT-A application induces a strong extracellular fibrotic response²⁸. The increase in collagen may also be confounded with the aging response. Indeed, it has been reported that aging results in increased slow MHC expression, and the saline group did show a slight reduction in type IIB and an increase in type IIX beginning at 6 months (Supplementary Table 3). Regarding titin,

we confirmed the minor changes previously reported for the giant intramuscular protein²², which accompanied changes in the elastic modulus of muscle cells²¹.

Because the current study was performed on normal muscle throughout the lifetime of the rat, they are not immediately applicable to the clinical use of BT-A on human spastic muscle contractures. This is because spastic muscle is already highly deranged with regard to its transcriptional profile²⁹, biomechanical properties^{30–32}, and sarcomere length^{33,34}. It is thus unknown whether the upper motor neuron lesion effects would be minimized or enhanced by blocking the NMJ. However, there are no animal models that recapitulate human spasticity. Importantly, the current data do not provide support for the generally accepted practice of treating spasticity in humans with BT-A injections repeated every 3–6 months³⁵. After 3–6 months in our model system, muscle fiber size has recovered to ~70% of normal and function to ~50% of normal. Based on the entirety of this study, we suggest that this altered function is due to inappropriate muscle innervation combined with moderate muscle atrophy in the time frame that would be considered reasonable for clinical redosing. This is because even only 50% of normal muscle function may be sufficient to create a joint contracture requiring further neurotoxin treatment.

Another reason that these data may not apply to human studies is that the injectate volume (100µL) is large relative to the size of the rat anterior compartment (1 mL⁸) but the dose (6.0 units/kg) is similar to that used in children. Much smaller relative volumes can be injected into large human muscles, and it is possible that these smaller volumes would produce results different than those observed in the current study.

In conclusion, this study demonstrated that muscles respond to a single BT-A injection with a rapid transcriptional response followed by a relatively long period of neuromuscular remodeling. While muscle fiber size and mass recover after a year, functional properties do not fully recover in that timeframe. The precise mechanism for the functional loss is unknown, but may be related to hampered NMJ function or impaired muscle fiber excitation-contraction coupling. Future studies are required to fully understand the mechanism of BT-A action. Such an understanding may improve our understanding of neuromuscular function and provide insights into the optimal clinical application of BT-A in medicine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BT	A-onabotulinumtoxinA neurotoxin
EDL	extensor digitorum longus

EHL	extensor hallucis longus
MHC	Myosin heavy chain
CSA	Muscle fiber cross, sectional area
NMJ	neuromuscular junction
SDS	PAGE-sodium dodecyl sulfate polyacrylamide gel electrophoresis
TA	tibialis anterior

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Figure 1.

(A) Body mass of animals injected with BT-A (filled bars) or saline (open bars) relative to the initial body mass. Note that mass increases and then decreases due to normal developmental and then aging processes. Data represent mean $\pm$ SEM on $n=90$ animal subjects (4–19/group; sample size shown on bar). *-significant difference between saline and BT-A injected groups at a given time point ($p<0.05$). Mass of TA muscles (B) contralateral to muscles injected with BT-A (filled bars) or saline (open bars) and (C) muscles injected with BT-A (filled bars) or saline (open bars). Note that no difference was observed between BT-A and saline (open bars) for contralateral control muscles, except for a spurious value at

3 months, indicating lack of a systemic effect. However, significant effects of BT-A were observed at early time points. Data represent mean $\pm$ SEM (4–19/group). *-significant difference between saline and BT-A injected groups at a given time point ($p < 0.05$).

Figure 2.

Representative histological sections of TA muscles at 1 week (A), 1 month (B), 3 months (C), 6 months (D), 12 months (E), and 18 months (F) after BT-A injection compared to saline-injected control (G). Fiber size heterogeneity and increased cellularity was observed at 1 month (B) and 3 months (C). At these time points, some fibers still retain normal size and morphology even though they are surrounded by highly atrophic fibers. Bar=100μm.

Figure 3.

Muscle fiber cross-sectional areas of TA muscles (A) injected with BT-A (filled bars) or saline (open bars) and (B) contralateral muscles. Note that muscle fiber area recovers in the BT-A group by 12 months of age. This contrasts with functional results (c.f. Fig. 5). Data represent mean $\pm$ SEM (4–12/group). *-significant difference between saline and BT-A injected groups at a given time point ($p < 0.05$).

Figure 4.

Dorsiflexion torque measured from animal subjects (A) injected with BT-A (filled bars) or saline (open bars) and (B) contralateral muscles. Note a negative torque 1 week after BT-A injection and, although a gradual recovery occurs, torque is still lower than saline-injected muscles after 12 months. Data represent mean $\pm$ SEM (4–19/group). *-significant difference between saline and BT-A injected groups at a given time point ($p < 0.05$).

Figure 5. Relationship between muscle fiber cross-sectional area (CSA) and dorsiflexion torque for the BT-A injected muscles. Each symbol represents a different time period. Regression coefficients and statistical analysis for each group are presented in Supplementary Table 4.