Published in final edited form as:

Anesth Analg. 2012 January ; 114(1): 102–109. doi:10.1213/ANE.0b013e31823526bf.

A Single Injection of Botulinum Toxin Decreases the Margin of Safety of Neurotransmission at Local and Distant Sites

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Abstract

Background—We tested the hypothesis that a single injection of botulinum toxin not only has local, but also distant effects on muscle function, biochemistry, and pharmacodynamics of atracurium.

Methods—Botulinum toxin (2.5U) was injected into the tibialis muscle of anesthetized rats (n=26). The contralateral side with no injection served to study distant effects. Control animals (n=25) received a saline injection. Neuromuscular function, pharmacology and expression of acetylcholine receptors (nAChRs) were evaluated in the tibialis at 0, 4 and 16 days after injection and compared to saline injected controls.

Results—On day 4, botulinum toxin caused complete paralysis of the tibialis, while its contralateral side showed a decrease in absolute twitch tension (1.8N [1.6; 1.9] vs. 3.0N [2.8; 3.1],

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DISCLOSURES:

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Contribution: This author helped design the study, conduct the study, analyze the data, and write the manuscript.

Attestation: Dr. Christiane G Frick has seen the original study data, reviewed the analysis of the data, approved the final manuscript, and is the author responsible for archiving the study files.

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Contribution: This author helped write the manuscript.

Attestation: PD Dr. Heidrun Fink has seen the original study data, reviewed the analysis of the data, and approved the final manuscript.

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Contribution: This author helped design the study, conduct the study, analyze the data, and write the manuscript.

Attestation: Prof. Dr. Manfred Blobner has seen the original study data, reviewed the analysis of the data, and approved the final manuscript.

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Contribution: This author helped design the study, conduct the study, analyze the data, and write the manuscript.

Attestation: Prof. Dr. J.A. Jeevendra Martyn has seen the original study data, reviewed the analysis of the data, and approved the final manuscript.

The authors declare no conflicts of interest.

Newton, $p < 0.05$). On day 16, muscle weakness was only present on the toxin-injected side where absolute twitch tension was decreased (0.6N [0.6; 0.7] vs. 3.4N [3.1; 3.7], $p < 0.05$). Tibialis mass was decreased on the toxin-injected side at day 4 (1.46mg/g [1.43; 1.48] vs. 1.74mg/g [1.72; 1.75], $P < 0.05$) and on day 16 (0.78mg/g [0.76; 0.79] vs. 1.73 mg/g [1.69; 1.77], $P < 0.05$). Effects distant from the site of injection were seen on day 16, when muscle atrophy was also present in the adjacent gastrocnemius and soleus muscles. Normalized to tibialis mass, specific twitch tension (tension/ g muscle) was reduced on the contralateral side at day 4 and on the toxin-injected side at day 16 relative to saline controls. At day 16, an increased sensitivity to atracurium was seen on the toxin-injected side, evidenced as a decreased ED_{50} (0.23mg/kg [0.13; 0.33] vs. 0.72 mg/kg [0.63; 0.82], $p < 0.05$), and a lower infusion rate (38 μ l/kg/min [32; 43] vs. 135 μ l/kg/min [126; 144], $p < 0.05$) together with a reduced plasma concentration requirement of atracurium (0.5 μ g/ml [0.4; 0.7] vs. 4.5 μ g/ml [3.8; 5.2], $P < 0.05$) to achieve a steady-state 50% reduction in baseline (absolute) twitch tension. ED_{50} of atracurium was also decreased on the contralateral side at day 16 relative to saline controls. The nAChRs in the tibialis were increased on the toxin-injected side to 123fmol/mg [115; 131] vs. 28fmol/mg [25; 29], ($p < 0.05$) in time-matched saline-injected controls at day 4 and to 378 [341; 413] vs. 27 fmol/mg [25; 29], ($p < 0.05$) at day 16.

Conclusions—Botulinum toxin has local and distant effects on muscle. The decrease in specific twitch tension indicates that the muscle atrophy alone cannot explain the functional changes; neuromuscular transmission is also impaired. An increased sensitivity to atracurium on the toxin-injected side, despite up-regulation of nAChRs, seems unique to botulinum toxin.

Introduction

Clostridium botulinum has well known effects at the neuromuscular junction (1,2). This exotoxin produces muscle paralysis by blocking the release of acetylcholine (ACh), leading to a denervation-like state including up-regulation of acetylcholine receptors (nAChRs) (2,3). Botulinum toxin (Botox®, Allergan Inc., Irvine, CA) is now extensively used for the treatment of muscle disorders such as torticollis, cerebral palsy and strabismus and more recently cosmetically, to reduce facial wrinkles or excessive perspiration (4-6). For each clinical application depending on the preparation, varying doses from 20 to 500 units of botulinum toxin have been used (7,8). In addition, botulinum toxin infection can occur in patients after traumatic injuries, musculo-skeletal tissue allografts, and in drug abusers (9,10).

Although the acute pre-junctional effects of botulinum toxin have been well studied, its acute post-junctional effects on local and distant muscle function and its interactions with neuromuscular blocking drugs are unknown. In fact, a case report indicated that a patient receiving botulinum toxin injection to the forehead exhibited unusual sensitivity to a neuromuscular blocking drug (11). This observation of increased sensitivity to neuromuscular blocking drugs seemed contradictory to expectations; up-regulation of nAChRs seen with botulinum toxin previously documented (1,2) should cause resistance to nondepolarizing neuromuscular blocking drugs as shown after burn injury or immobilization and not increased sensitivity (12).

Clinical studies indicate that the effects of botulinum toxin last approximately 3 months (1,2). Therefore, our previous study (13) evaluated the effects of botulinum toxin beyond the three months. We demonstrated that a single injection of increasing doses (0.625U, 2.5U and 10U) of botulinum toxin injected into the tibialis muscle caused dose-dependent long-term (128 days after injection) functional, pharmacological and biochemical changes at the neuromuscular junction (13). However, the onset and early effects of botulinum toxin remain unclear. The current study tested the hypothesis that a single local injection of botulinum toxin leads to alterations in neuromuscular transmission at local and distant sites.

The hypothesis was tested by examining the “early” effects of botulinum toxin on neuromuscular transmission during nerve evoked tension development, with and without the neuromuscular blocking drug, atracurium at 0, 4 and 16 days after injection (infection) of toxin into a single (tibialis) muscle.

Methods

Animal Model, Group Assignment and Botulinum Toxin Injection

Institutional approval for this study was obtained. Male Sprague-Dawley rats (245-320g) were used. Animals were allowed to accommodate to the standard conditions of our animal facility with free access to rat chow and water for at least a week. Animals were allocated to study groups based on time (0, 4 and 16 days) of the functional study after injection and type of injection (toxin or saline). Botulinum toxin (Botox®, Allergan Inc., Irvine, California) was reconstituted with 0.9% sterile saline. The stock solution of 100U was further diluted with 0.9% sterile saline. On day 0, the experimental group received an injection of 2.5U of the toxin into the tibialis muscle, while control animals received an equivalent volume of saline (Figure 1).

For the injection, the rats were anesthetized with pentobarbital (60mg/kg i.p.), the limb shaved and disinfected. The total volume (0.5ml) of diluted botulinum toxin or saline was aliquoted into two equal parts (0.25ml) and then injected into the medial and lateral aspects of the middle of the tibialis muscle belly, where the neuromuscular junctions are usually located. The contralateral (noninjected) side that received no injection served to study the distant effects of the toxin. The contralateral (noninjected) side of saline animals served as naive control. Following the injections, the animals were returned to their cages after recovery from anesthesia.

Anesthesia and Vital Parameters

For the neuromuscular functional and pharmacological studies, the animals were re-anesthetized with pentobarbital (60mg/kg i.p.) on the experimental day, tracheotomized and their lungs mechanically ventilated. The right jugular vein was catheterized for drug and fluid administration. The right carotid artery was cannulated to measure arterial blood pressure and perform blood gas analyses. Heart rate, mean arterial blood pressure and body temperature were continuously monitored to ensure stable hemodynamic conditions throughout the experiment. Arterial PaO₂, PaCO₂, and acid-base status were intermittently measured and corrected if necessary. Anesthesia was maintained with repetitive doses of pentobarbital based on cardiovascular signs of inadequate anesthesia. Rats were excluded from the experiment if they were hemodynamically unstable (mean arterial blood pressure < 80 mm Hg) or if the blood gases throughout the experiment were not within the predefined ranges (PaO₂ > 100 mm Hg; pH 7.36-7.44; PaCO₂ = 36-44 mmHg; base excess of -2 ± 2mEq).

Evaluation of Neuromuscular Function

Neuromuscular function was monitored by evoked mechanomyography using a nerve stimulator (NS252, Fisher & Paykel, Health Care, Irvine, CA) along with a Grass Force FT03 transducer (Grass Instruments, Quincy, MA). For this purpose, rats were placed in the dorsal recumbent position. The tendon of the insertion of tibialis muscle on each side was surgically exposed, severed and then individually attached to separate transducers. Both sciatic nerves were exposed at the thigh and stimulation electrodes were attached for measurement of nerve-mediated contraction of the tibialis muscle. To ensure a force vector control, each knee was stabilized rigidly with a clamp. A preload of 0.5 N (Newton) was applied to yield maximal evoked isometric contractions. The nerve-evoked tensions of the

respective tibialis muscle were recorded via a Grass P122 amplifier and displayed using the Grass Polyview Software (Grass Instruments, Quincy, MA).

Train-of-four (TOF) and tetanic muscle tensions were determined to assess muscle strength (absolute twitch and peak tetanic tension) and performance of repetitive work (TOF ratio, tetanic fade). Initially, during supramaximal TOF stimulation at 2Hz, repeated every 20 seconds, baseline twitch tension was stabilized over a period of 10 minutes. At the end of the 10-minute period of stabilization, the mean values of ten T1 twitches (first twitch of the TOF stimulation pattern) were calculated and recorded as the absolute twitch tension. To calculate TOF ratio, the T4, the fourth twitch of the TOF stimulation pattern was related to the T1, the first twitch of the TOF stimulation. After the initial 10 minutes of TOF stimulation, we applied a single tetanic contraction. A tetanic contraction occurs when a motoneuron has been intensely repetitively stimulated. In this study, tetanic tension was assessed once by a 50 Hz (50 stimuli per second) stimulation for 5 seconds to calculate the peak tetanic tension and fade associated with this stimulus. The initial peak was taken as peak tetanic tension. Tetanic fade was calculated as $([\text{initial peak contraction} - \text{final contraction at the end of 5 sec}] * 100) / (\text{initial peak contraction})$. All forces were measured in [N].

After an interval of 30 min with ongoing TOF stimulation, to allow muscle recovery from the effects of the preceding tetanus, the potency of atracurium, a nondepolarizing neuromuscular blocking drug, was tested on both sides by the cumulative dose-response method. Bolus doses of atracurium were given IV in increments of 0.1- 0.4 mg/kg until the first twitch height (T1) of the TOF was below approximately 5% of the baseline twitch tension (>95% twitch depression) on both sides. Each incremental dose was only given when the previous dose had produced maximal effect, as indicated by three equal consecutive T1 twitches. After the last dose of atracurium was given, the T1 value was allowed to recover to baseline values. Complete recovery of T1 values was assumed when the measured T1 was >95% of baseline T1 twitch height. Subsequently, a continuous infusion of atracurium was started, and the infusion rate was adjusted to achieve an approximately 50±2% T1 inhibition on the botulinum toxin or saline injected side. After 10 minutes of stable 50% T1 inhibition on the injected side, a pseudo steady-state condition was assumed to be present between plasma and the neuromuscular junction. At this point, the twitch height on the noninjected side was also noted and one ml of heparinized blood was withdrawn, for later determination of plasma concentrations of atracurium for 50% depression on the injected side. The blood was immediately transferred to an Eppendorff tube containing 20µl 1 M H₂SO₄ and centrifuged (3,500 rpm, 10 min, 4°C). The plasma (0.2 ml portions), aliquoted into Eppendorff tubes containing 0.8 ml 15mM H₂SO₄ was immediately frozen at -80°C. After blood sampling, both tibialis, gastrocnemius, and soleus muscles on both sides were harvested, weighed, and snap frozen on dry ice and stored at -80°C for later determination of nAChRs concentrations.

Acetylcholine Receptor Assay

The nAChR protein expression in the tibialis muscles was assayed using the ¹²⁵I-α-bungarotoxin binding assay, as previously described (14), and expressed as femtomol nAChR per milligram protein. The protein concentration of the muscle extract was assayed using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Atracurium Plasma Levels Assay

The plasma concentration of atracurium was determined by high performance liquid chromatography (HPLC) as described previously (15). Briefly, 20µg verapamil as an internal standard was added to the pretreated samples (200µl serum + 800µl 15 mM H₂SO₄).

Samples were deproteinized using 1 ml acetonitrile and centrifuged at 5000g. Fifty μ l of the supernatant was injected into the HPLC column (RP18, LiChrospher, 5 μ m, 100 mm, 4.6 ID; Merck, Darmstadt, Germany). Atracurium was separated using a linear gradient elution system: from A to 100% B in 8 min (A [23.5 ml acetonitrile, 5 ml methanol, and 0.03 M K_2HPO_4 , 57.5 ml, adjusted to pH 5], B [35.5 ml acetonitrile, 15 ml methanol, and 0.1 M K_2HPO_4 , 47.5 ml, adjusted to pH 5], flow rate 1.7 ml/min, fluorescence detection at 240 nm excitation and 320 nm emission). A calibration solution was prepared from authentic substances with 5.0, 15.0, and 24.0 μ g/ml atracurium and 1.0, 2.0, and 3.0 μ g/ml laudanosine in 0.005 M H_2SO_4 , pH 2.5. The plasma clearance of atracurium during steady-state conditions was calculated by the equation: clearance = infusion rate/plasma concentration.

Data and Statistical Analyses

In a previous study, saline-injected animals had an absolute twitch tension of $2.8N \pm 0.5N$ (mean $\pm$ SD) at 128 days after injection (13). We assumed that a reduction of absolute twitch tension (ΔF) to at least 30% after toxin injection to be relevant resulting in a critical difference $D = \Delta F/SD = 0.8N / 0.5N = 1.6$. With errors of 0.05 for the type one error and 0.2 for the type two error, 6 animals per group are necessary for statistical evaluation. Although a hierarchical statistical model was chosen to address the multiple comparisons, we decided to increase the number of animals to 8 per group. Because of potential attrition due to various factors such as hemodynamic and metabolic instability during the functional studies, we started with 9-10 animals per group in our randomization schedule. If one animal had to be withdrawn, an extra animal was added to the respective group.

All values given in the abstract and the tables are expressed as means and 95% confidence intervals (CI). In the results section, the mean differences, their CIs and the p-values are given for our most important findings. Values were compared by 2-way repeated measurement analyses of variance, using time, injected agent (between groups) and leg (within group) as independent factors. If one effect proved to be significant, post hoc testing was performed with paired and unpaired t-tests ($p < 0.05$) respectively. Assuming that the twitch response or the relationship of neuromuscular block to the dose of atracurium was governed by the Hill equation, linear regressions of the degree of block in logit scale and the respective cumulative dose of atracurium in log scale were calculated for each leg. The transformed values were statistically evaluated by an analysis of co-variance for repeated measurements using time (between groups) and leg (within group) as independent factors and characterized by the effective doses for a 50% neuromuscular block (ED_{50}) and the 95% CI. Within the tables and figures the dose-response relation is presented in linear scale by retransformation of the respective means and confidence limits. Therefore, the presented 95% CI are not symmetrical to their means. Post hoc testing was performed with paired or unpaired t-tests ($p < 0.05$). Statistical tests were calculated using SPSS for MAC Version 18.0 (IBM Corporation, Somers, NY).

Results

Model Stability

Thirty-three animals were injected in the toxin group (day 0, $n = 9$; day 4, $n = 8$; day 16, $n = 16$) and 28 animals in the saline group (day 0, $n = 11$; day 4, $n = 9$; day 16, $n = 8$). All animals survived the botulinum toxin or saline injection. Their gain in body weight did not differ between groups (5 ± 1 g). Ten rats were excluded from the experiment during the functional studies due to hemodynamic instability (mean arterial blood pressure < 80 mm Hg) and metabolic instability (values not within the predefined ranges: $PaO_2 > 100$ mm Hg; pH 7.36-7.44; $PaCO_2 = 36$ -44 mmHg; base excess of -2 ± 2 mEq). In detail, one rat from the 2.5U botulinum toxin group was excluded on day 0, six animals from the 2.5U botulinum

toxin group on day 16, and three rats from the saline group on day 0. The final statistical analysis included 26 animals in the botulinum toxin group (day 0, n = 8; day 4, n = 8; day 16, n = 10) and 25 animals in the saline group (day 0, n = 8; day 4, n = 9; day 16, n = 8).

Muscle Contractility and Fatigability

The muscle function data are summarized in Table 1. On day 4, the toxin injection induced complete neuromuscular paralysis of the respective tibialis muscle, so that no muscle contraction could be evoked on that side. At this time, the absolute twitch (1.2N [0.6N; 1.8N], $p=0.001$) and peak tetanic (3.7N [1.6N; 5.8N], $p=0.002$) tensions were significantly decreased on the side contralateral to injection, without affecting muscle performance (TOF ratio or tetanic fade). On day 16, impaired muscle function was seen on the toxin-injected side where absolute twitch (2.8N [1.9N; 3.7N]; $p<0.001$) and peak tetanic (6.9N [5.1; 8.7], $p<0.001$) tensions were significantly depressed (Table 1). Normalized to muscle mass, the specific twitch (2.0N/g [0.8N/g; 3.3N/g], $p=0.003$) and specific tetanic (6.1N/g [1.7N/g; 10.5N/g], $p=0.010$) tensions (tensions in N per g tibialis muscle mass) were also significantly decreased on the contralateral side on day 4. At day 16, specific twitch (3.4N/g [1.7N/g; 5.1N/g], $p=0.001$) and specific tetanic (8.1N/g [5.2N/g; 10.9N/g], $p<0.001$) tension were reduced on the toxin-injected side at day 16 relative to saline-injected time matched controls (Table 1). However, our study design allows further comparisons, as shown in Table 1.

Atracurium Pharmacodynamics

The atracurium pharmacodynamics data are summarized in Table 2. On day 0, the ED_{50} of atracurium in the toxin-injected leg was significantly increased. However, during steady-state infusion of atracurium to achieve 50% depression of T1 values on the toxin-injected side (day 0), no differences were observed in the slopes of the dose-response curves, the infusion rates and the plasma concentrations of atracurium. At that time, the twitch height on the contralateral side was significantly higher (less depressed). On day 4, atracurium pharmacodynamics could not be determined on the injected side due to complete paralysis of the tibialis muscle. On the contralateral side, where muscle weakness was seen at that time, the ED_{50} values of atracurium were significantly reduced compared to the ED_{50} of the respective leg on day 0 (Table 2); the slopes did not differ between groups and sides. At day 16, the slope (3.1 [2.5; 3.7], $p<0.001$) of the dose-response curve was significantly reduced on the toxin-injected side (Table 2). At this time, the dose-response curve for atracurium was significantly shifted to the left, resulting in lower ED_{50} values not only on the toxin-injected side, but also on its contralateral side (Figure 2). Increased sensitivity to atracurium was also evidenced as a decreased infusion rate (98 μ l/kg/min [68 μ l/kg/min; 127 μ l/kg/min], $p<0.001$) and a lower plasma concentration of atracurium (4.0 μ g/ml [2.4 μ g/ml; 5.5 μ g/ml], $p<0.001$) to achieve steady-state 50% depression of T1 values on the toxin-injected side (day 16), (Table 2). Therefore, the twitch height (40% [25%; 56%], $p<0.001$) on the contralateral side was significantly higher than the 50% twitch height achieved on the injected side. Changes in ED_{50} values or slopes of the dose-response curve on day 0 and 16 are indicated (injected or contralateral side) in the experimental group compared to the respective side of their time-matched saline-injected animals (group effect). Additional significant changes are shown in Table 2.

Muscle Mass and nAChR concentrations

The muscle mass data are summarized in Table 3. On day 0, tibialis muscle mass was unchanged between sides and groups (Table 3). Later, there was a time-dependent significant decline in tibialis muscle mass to 80% (day 4) and 44% (day 16), respectively, on the toxin-injected side compared to the contralateral leg. At day 16, muscle atrophy was also seen in the adjacent gastrocnemius and soleus muscles (Table 3). The concentrations of

membrane nAChRs in the tibialis muscle (day 4: 31 fmol/ mg protein [16 fmol/mg; 60 fmol/ mg], $p < 0.001$ and day 16: 365 fmol/mg protein [83 fmol/mg; 1600 fmol/mg], $p < 0.001$) were significantly increased in a time dependent manner compared to the contralateral leg and to saline injected controls (Figure 3).

Discussion

This study confirms the hypothesis that a single intramuscular injection of botulinum toxin has local and distant postjunctional effects. On a functional level, time-dependent neuromuscular changes from no paralysis at 4 hours, complete paralysis at day 4 to severe muscle weakness with increased fatigability (fade) at 16 days were observed. Pharmacologically, an increased sensitivity to the neuromuscular effects of atracurium was seen together with a profound up-regulation of nAChRs at day 16. Both functional and pharmacological changes were also present on the contralateral side. All of these findings suggest that botulinum toxin decreases the margin of safety of neurotransmission and muscle contraction by prejunctional and postjunctional mechanisms (see below for explanations).

Botulinum toxin binds to nerve terminals and impairs exocytosis of ACh vesicles (16), leading to flaccid paresis with intact sensation (1,2). Our functional studies, performed 4 hours after injection, showed that the dose used does not immediately affect muscle function. Four days later, however, the single injection of toxin caused complete paralysis of the injected muscle. Some recovery effects were seen within two weeks after injection. However, despite the presence of nerve-mediated contraction on day 16, muscle tension was severely depressed. The reduction in specific tensions on the contralateral side at day 4 and on the ipsilateral side on day 16 relative to saline-injected time-matched controls suggest that, in addition to muscle atrophy, neurotransmission is impaired most likely due to the toxin-related decreased prejunctional release of ACh (1-3). This finding contrasts with observations in our previous study examining the long-term (128 days) effects of different doses (0.625U, 2.5U and 10U) of botulinum toxin on neuromuscular transmission. In that study, no differences were seen in specific muscle tensions suggesting that the prejunctional effects disappear by 128 days and muscle atrophy was the predominant reason for impaired muscle function at that time (13). Although we did not measure junctional nAChRs, total nAChRs on muscle membrane were in fact increased on day 16 and usually included up-regulation of fetal nAChRs. The fetal nAChRs depolarize even at very much lower (1/10th) concentrations of ACh (12,17). The de novo increase in AChRs, however, could not compensate for the attenuated ACh release, evidenced as decreased function. Additionally, a reduction in both specific tensions was also seen on the contralateral leg of toxin-injected animals at day 4 suggesting systemic spread of toxin to distant sites. At day 16, the distant effects could only be seen in the soleus and gastrocnemius muscles on the injected side, evidenced as muscle atrophy.

The decreased release of ACh leads to a dramatic up-regulation of nAChRs in a time-dependent manner. Up-regulation of nAChRs at the muscle membrane has been shown to increase the dose requirements of a competitive antagonists (e.g., atracurium) (12,17,18). In this study, however, there was increased sensitivity to the effects of atracurium on the toxin-injected side at day 16, in association with a smaller slope and profound up-regulation of nAChRs. The smaller slope suggests decreased affinity of atracurium to nAChRs at the junction (13,19). This is probably related to the expression of denovo nAChRs. Everything being equal, a smaller slope would shift the dose-reponse curve to the right, in case of an unchanged intercept. However, the ED is shifted to the left at day 16. It seems, therefore, that the prejunctional effects of the toxin dominated, resulting in increased sensitivity to atracurium. These findings contrast with previous studies where resistance has been reported

during up-regulation of AChRs induced by infection, burn, immobilization or denervation (12,20,21). Surprisingly, the ED₅₀ was increased on the toxin-injected side at day 0. Since this cannot be related to an altered expression of nAChRs, one can speculate if this finding could be due to increased ACh levels resulting from a botulinum toxin-induced decreased activity of acetylcholinesterase at that time (22). We did not, however, measure ACh levels or activity to confirm this hypothesis.

The main limitation of this study is the fact that the effects of botulinum toxin were studied after injection of a single dose of toxin into the tibialis muscle. Although the toxin was injected equally into the medial and lateral aspects of the tibialis muscle belly, one does not know if the toxin really disperses equally within the neuromuscular junction, in the tibialis muscle and the rest of the body. We did see systemic effects on the contralateral side suggesting the spread of toxin. On the other hand, it would be challenging to study systemic infection as seen in patients in an animal model, due to the difficulties in maintaining an animal with generalized paralysis for prolonged periods of time.

The clinical implications of this study are as follows: the effects of botulinum toxin are not visible within a few hours of infection and follow a time course from complete paralysis to severely depressed muscle function. The neuromuscular effects can be local as well as distant. An increased sensitivity to the effects of nondepolarizing neuromuscular blocking drugs has to be anticipated, despite up-regulation of nAChRs. If botulinum toxin has been injected into a certain area for therapeutic or cosmetic purposes, the twitch pad applied to the area of toxin injection to monitor neuromuscular function during administration of neuromuscular blocking drugs may provide incorrect information regarding the rest of the body. In view of the distant effects (e.g., contralateral side), neuromuscular monitoring even at other sites (e.g., diaphragm) may not offer accurate reflection of the state of paralysis in the rest of the body. This is consistent with the observation in a case report where a twitch pad applied to the forehead in a patient who had botulinum toxin injection to his orbicularis oculi mislead the anesthesiologist's assessment of neuromuscular block in the abdomen (11).

Acknowledgments

Funding: Supported by Grants (GM 031569, GM 055082, GM 21500-Project IV) from the National Institutes of Health, Bethesda, Maryland, USA, and Shriners Hospital Research Philanthropy, Tampa, Florida, USA to J.A.J. Martyn.

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Figure 1. Flow diagram of our planned experiments

Thirty-three animals were injected in the toxin group (day 0, n = 9; day 4, n = 8; day 16, n = 16) and 28 animals in the saline group (day 0, n = 11; day 4, n = 9; day 16, n = 8). Due to an exclusion of 10 rats because of hemodynamic and metabolic instability on the day of their functional studies, the final statistical analysis included 26 animals in the botulinum toxin group (day 0, n = 8; day 4, n = 8; day 16, n = 10) and 25 animals in the saline group (day 0, n = 8; day 4, n = 9; day 16, n = 8).

Figure 2. Cumulative dose-response curves to atracurium [mg/kg] at day 16

At day 16 after injection of botulinum toxin, pharmacodynamics of atracurium were evaluated using the cumulative dose-response curve method. The dose-response curve for atracurium was shifted to the left both on the toxin-injected and contralateral sides, resulting in significantly ($p < 0.05$) lower ED_{50} when compared to the saline-injected controls. The slope was significantly ($p < 0.05$) smaller on the toxin-injected side compared to controls and contralateral side. The bars represent the confidence intervals.

Figure 3. Acetylcholine receptor expression (nAChR) [fmol/ mg protein] in the tibialis muscle
At 0, 4 and 16 days after botulinum toxin or saline injection, nAChRs expression on the injected, as well as on the contralateral side, which received no injection were measured. Separate, animals with saline injection on one side and its contralateral naive side served as controls. The concentrations of membrane nAChRs in the tibialis muscle were significantly ($p<0.05$) increased at day 4 and 16 compared to that of the contralateral leg, saline-injected leg and naive controls. Number of nAChRs was significantly ($p<0.05$) higher in the toxin-injected side on day 16 when compared to the toxin-injected side on day 4. Expression of nAChRs was also significantly ($p<0.05$) increased in the toxin-injected side on day 4 and 16 when compared to the toxin-injected side on day 0. Values are expressed as means $\pm$ confidence intervals.

Table 1

a. Muscle function after botulinum toxin or saline.

Side	Day 0 saline	2.5U botox	Day 4 saline	2.5U botox	Day 16 saline	2.5U botox
Absolute twitch tension [N]	3.1	2.8	2.9		3.4	0.6 ^{***†‡}
	injected	[2.8; 3.3]	[2.6; 3.1]	[2.8; 3.0]	paralysis ^{***†}	[3.1; 3.7]
						[0.6; 0.7]
	contralateral	3.0	2.7	3.0	1.8 [#]	3.4
Specific twitch tension [N/g]		[2.7; 3.2]	[2.5; 2.9]	[2.8; 3.1]	[1.6; 1.9]	[3.1; 3.6]
	injected	5.2	5.6	5.9		2.1 ^{***†‡}
		[4.8; 5.6]	[5.2; 5.9]	[5.7; 6.1]	paralysis ^{***†}	[1.9; 2.4]
	contralateral	5.0	5.2	5.9	3.9 [#]	5.0
Peak tetanic tension [N]		[4.6; 5.4]	[4.8; 5.5]	[5.7; 6.1]	[3.6; 4.2]	[4.7; 5.4]
	injected	8.1	6.9	8.5		1.7 ^{***†‡}
		[7.4; 8.8]	[6.1; 7.6]	[8.1; 9.0]	paralysis ^{***†}	[1.6; 1.8]
	contralateral	8.0	7.1	8.5	4.8 [#]	9.4 [‡]
Specific tetanic tension [N/g]		[7.3; 8.7]	[6.3; 7.9]	[8.1; 9.0]	[4.3; 5.3]	[8.8; 10.0]
	injected	13.7	13.2	17.5		5.9 ^{***†‡}
		[12.6; 14.7]	[12.0; 14.4]	[16.6; 18.4]	paralysis ^{***†}	[5.6; 6.1]
	contralateral	13.4	13.5	16.9	10.8 [#]	14.1
b. Muscle fatigability after botulinum toxin or saline.		[12.3; 14.4]	[12.3; 14.8]	[16.2; 17.6]	[9.4; 12.2]	[13.3; 14.9]

b. Muscle fatigability after botulinum toxin or saline.

Side	Day 0 saline	2.5U botox	Day 4 saline	2.5U botox	Day 16 saline	2.5U botox
Train-of-Four- Ratio	0.98	0.98	0.99		0.99	0.93 [#]
	injected	[0.98; 0.99]	[0.97; 0.99]	[0.99; 1.00]	paralysis	[0.98; 0.99]
						[0.93; 0.94]
	contralateral	0.99	0.99	0.99	0.98	0.99
Tetanic Fade [%]		[0.99; 0.99]	[0.98; 0.99]	[0.98; 0.99]	[0.97; 0.98]	[0.98; 1.0]
	injected	10.2	8.6	6.4		24.5 ^{**}
					paralysis	

Side	Day 0 saline	2.5U botox	Day 4 saline	2.5U botox	Day 16 saline	2.5U botox
contralateral	[8.0; 12.5]	[7.7; 9.5]	[5.9; 6.7]		[4.1; 5.2]	[20.0; 29.0]
	5.1	6.0	6.8	8.5	4.2	7.1
	[4.3; 5.9]	[5.0; 7.0]	[6.2; 7.4]	[7.6; 9.4]	[3.1; 5.3]	[5.8; 8.3]

All values are expressed as means and confidence intervals in parenthesis

* p<0.05 versus contralateral leg (R-L-Comparison);
p< 0.05 versus saline group (group effect);
[†] p< 0.05 versus day 0;
[‡] p<0.05 versus day 4.

Table 2

Atracurium pharmacodynamics, steady-state.

	Side	Day 0 saline	2.5U botox	Day 4 saline	2.5U botox	Day 16 saline	2.5U botox
ED ₅₀ [mg/kg] of atracurium	injected	0.73 [0.59; 0.91]	0.90 ^{##} [0.78; 1.05]	0.67 [0.58; 0.78]	paralysis	0.72 [0.63; 0.82]	0.23 ^{##†} [0.13; 0.33]
	contralateral	0.68 [0.52; 0.87]	0.76 [0.62; 0.90]	0.65 [0.55; 0.76]	0.60 [†] [0.55; 0.66]	0.70 [0.61; 0.83]	0.58 ^{##†} [0.49; 0.70]
Slope of dose-response curve	injected	4.3 [4.0; 4.7]	4.5 [3.6; 5.5]	5.4 [4.8; 6.0]	paralysis	4.9 [4.7; 5.2]	1.8 ^{##†} [1.4; 2.3]
	contralateral	4.6 [3.7; 5.4]	4.7 [3.3; 6.0]	5.8 [5.1; 6.5]	5.4 [4.9; 6]	4.9 [4.6; 5.3]	4.7 [4.0; 5.4]
Infusion rate [μ l/kg/min] (at 50% paralysis)	injected	146 [136; 155]	115 [106; 125]	124 [117; 132]	paralysis	135 [126; 144]	38 ^{##†} [32; 43]
	injected	3.4 [3.2; 3.7]	4.1 [3.4; 4.8]	3.7 [3.5; 4.0]	paralysis	4.5 [3.8; 5.2]	0.5 ^{##†} [0.4; 0.7]
Neuromuscular block [%] (at steady-state 50% T1 inhibition on the toxin-injected side)	contralateral	50 [41, 54]	26 [6;43] ^{##}	50 [32, 61]	paralysis	50 [33, 61]	5 [1;18] ^{##}

All values are expressed as means and confidence intervals in parenthesis

* p<0.05 versus contralateral leg (R-L-Comparison);

p<0.05 versus saline group (group effect);

† p<0.05 versus day 0.

The effective doses for a 50% inhibition of T1 twitch height (ED50) as well as the 95% confidence intervals were calculated by simple retransformation of the respective means and confidence limits into linear scale.

Table 3

Muscle mass per body mass.

	Side	Day 0 saline	2.5U botox	Day 4 saline	2.5U botox	Day 16 saline	2.5U botox
Tibialis muscle mass [mg/g]	injected	1.87 [1.84; 1.90]	1.73 [1.70; 1.75]	1.74 [1.72; 1.75]	1.46 ^{##†} [1.43; 1.48]	1.73 [1.69; 1.77]	0.78 ^{##†} [0.76; 0.79]
	contralateral	1.85 [1.83; 1.88]	1.76 [1.73; 1.78]	1.69 [1.66; 1.72]	1.84 [1.80; 1.88]	1.73 [1.69; 1.78]	1.77 [1.74; 1.81]
	injected	5.22 [5.13; 5.30]	4.67 [4.58; 4.75]	4.77 [4.67; 4.86]	3.95 [3.69; 4.21]	4.55 [4.42; 4.68]	3.02 ^{##†} [2.93; 3.12]
	contralateral	5.18 [5.10; 5.26]	4.63 [4.54; 4.72]	4.62 [4.55; 4.69]	4.79 [4.71; 4.86]	4.65 [4.56; 4.74]	4.59 [4.50; 4.67]
Gastrocnemius muscle mass [mg/g]	injected	0.41 [0.40; 0.43]	0.42 [0.39; 0.45]	0.43 [0.42; 0.44]	0.36 [0.31; 0.41]	0.36 [0.34; 0.38]	0.24 ^{##†} [0.23; 0.26]
	contralateral	0.44 [0.42; 0.45]	0.42 [0.41; 0.43]	0.42 [0.41; 0.44]	0.44 [0.40; 0.47]	0.35 [0.33; 0.37]	0.40 [0.38; 0.41]

All values are expressed as means and confidence intervals in parenthesis

* p<0.05 versus contralateral leg (R-L-Comparison);

p< 0.05 versus saline group (group effect);

† p< 0.05 versus day 0;

‡ p<0.05 versus day 4.