

## Long-term Effects of Botulinum Toxin on Neuromuscular Function

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**Background:** Recent reports indicate increased incidence of *Clostridium botulinum* infections, particularly among drug abusers and tissue allograft recipients. Botulinum toxin also has potential application in biochemical warfare. The neurotoxin-induced paralysis often requires mechanical ventilation with and without muscle relaxants. The authors investigated the long-term effects of botulinum toxin on muscle function, expression of nicotinic acetylcholine receptors (nAChRs), and their interaction with muscle relaxant, atracurium.

**Methods:** Rats (n = 30) were injected with varying doses (0.625, 2.5, and 10 U) of botulinum toxin into the tibialis muscle. Control animals (n = 9) received an equivalent volume of saline. At 128 days after injection, neuromuscular function, pharmacodynamics of atracurium, and nAChRs were evaluated.

**Results:** Nerve-evoked tensions, including tetanic tension and muscle mass, were decreased on the toxin-injected side in a dose-dependent manner relative to saline-injected controls as well as the contralateral side. Specific muscle tension and specific tetanic muscle tension (tensions/muscle mass) were not reduced. The ED<sub>10</sub> of atracurium was reduced, the ED<sub>50</sub> was unchanged, and the ED<sub>90</sub> was increased in the highest (10-U) dose of toxin group. The atracurium plasma concentration to maintain a steady state 50% paralysis was significantly reduced in the 10-U toxin group. The nAChR concentrations in the tibialis muscle were significantly increased in a dose-dependent manner in all experimental groups.

**Conclusion:** Botulinum toxin causes dose-dependent long-term neuromuscular changes. The loss of tension generating capacity is almost exclusively related to muscle atrophy, because the specific tension did not change. The decreased ED<sub>10</sub>, unaltered ED<sub>50</sub>, and increased ED<sub>90</sub> to atracurium suggest its

interactions with different isoforms of receptors having varying sensitivity to atracurium. The absence of fade, despite the persistent botulinum toxin-induced denervation (increased nAChRs), suggests that the up-regulated nAChRs may have compensated for the prejunctional effects of botulinum toxin.

RECENT reports indicate increasing incidence of clostridial infections in both Canada and the United States with high morbidity and mortality.<sup>1,2</sup> Several deaths due to *Clostridium sordelli* have been reported after medically induced abortion by mifepristone.<sup>3</sup> Almost all clostridial infections, including *C. sordelli*, *C. difficile*, *C. perfringens*, *C. tetani*, and *C. botulinum*, to name a few, have greater or lesser effects on neurotransmission in skeletal muscle.<sup>4,5</sup> Of these, *C. botulinum*, which releases botulinum toxin, has the most profound effects on skeletal muscle neurotransmission. Infection with *C. botulinum* is particularly common after traumatic injuries, among drug abusers,<sup>6–8</sup> and after musculoskeletal tissue allografts.<sup>9,10</sup> Infant botulism is the most common form of human botulism in the United States.<sup>11</sup> The spores of *C. botulinum* germinate and colonize the wound or gut, where they release the toxin into the bloodstream. The circulating toxin binds to nerve terminals, preventing the release of acetylcholine leading to neuromuscular paralysis.<sup>11–13</sup> The toxin itself also has potential application in biochemical warfare due to its extreme toxicity. The United States, the former Soviet Union, and Iraq, at one time or other, have tested this toxin for potential use as a biologic weapon.<sup>14</sup>

Botulinum toxin is also clinically used for several muscle disorders, including spasmodic torticollis, dysphonia, cerebral palsy, strabismus, anorectal sphincter abnormalities, and myofascial pain.<sup>15–17</sup> In recent years, the fastest growing use for botulinum toxin has been in cosmetic and plastic surgical applications and treatment of excessive sweating, e.g., hand sweating. Normally, the doses used for treatment of these disorders are extremely small, with localized effect of short duration. However, one serious drawback of even small doses is the mild to moderate, usually short-term local and distant neuromuscular effects.<sup>18–20</sup> With *C. botulinum* infection, however, the effect on the muscle can be more profound depending on the severity of the infection and the toxin levels released.<sup>12,13,21</sup> The end result can be neuromuscular dysfunction of varying duration. The paralysis produced, due to inhibition of the release of acetylcholine, can lead to a denervation-like state.<sup>22,23</sup> The long-term

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effects of clostridial toxin on muscle function and pharmacology have not been evaluated.

We hypothesized that the long-term effects of clostridial botulinum infection on the neuromuscular junction, simulated by injection of botulinum toxin, would be dose dependent with effects not only on muscle function, but also on pharmacology of muscle relaxants and expression of nicotinic acetylcholine receptors (nAChRs). In the view of the difficulties in maintaining an animal with generalized paralysis for prolonged periods of time, the effects of botulinum toxin on the neuromuscular junction were studied at 128 days after the injection (infection) of toxin into a single muscle (tibialis). We found that botulinum toxin effected dose-dependent neuromuscular changes, including muscle weakness, muscle mass loss, increased number of acetylcholine receptors, and, unexpectedly, a trimodal sensitivity to nondepolarizing muscle relaxant, atracurium.

## Materials and Methods

### *Animal Model, Group Assignment, and Botulinum Toxin Injection*

Approval was obtained from the Subcommittee on Animal Care Research at the Massachusetts General Hospital, Boston, Massachusetts. Male Sprague-Dawley rats, weighing 200–260 g, 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.

The animals were randomly allocated to three experimental groups, each group receiving differing doses of botulinum toxin (0.625 U,  $n = 12$ ; 2.5 U,  $n = 12$ ; and 10 U,  $n = 12$ ). Botulinum toxin (Botox<sup>®</sup>; Allergan Inc., Irvine, CA) was reconstituted with 0.9% sterile saline. The stock solution of 100 U was further diluted with 0.9% sterile saline according to the manufacturer's instructions. Controls ( $n = 12$ ) received an equivalent volume of saline.

For the injection, the rats were anesthetized with pentobarbital (60 mg/kg intraperitoneal), the limb shaved and disinfected. One group of animals received injection of botulinum toxin only. The total volume (0.5 ml) of diluted botulinum toxin was aliquoted into two equal parts (0.25 ml) and then injected into the medial and lateral aspects of the middle of the tibialis cranialis muscle belly, where the neuromuscular junction is usually located. The contralateral (noninjected) side that received no injection served to study the distant effects of the toxin. A separate control group of animals received an equivalent volume of saline using the same injection technique. The contralateral (noninjected) side that received no injection served as naive control. After injection of toxin or saline, the animals were monitored until recovery from anesthesia, and then returned to their

cages. Clinical studies indicate that the effects of botulinum toxin last for approximately 3 months. This study, therefore, looked at the neuromuscular effects beyond that period, at 128 days after injection.

### *Anesthesia and Vital Parameters*

Anesthetized with pentobarbital (60 mg/kg intraperitoneal) at 128 days, the animals were tracheostomized and ventilated using a Harvard ventilator. 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 and mean arterial pressure were continuously monitored to ensure stable hemodynamic conditions throughout the experiment. Arterial oxygen tension ( $P_{aO_2}$ ), arterial carbon dioxide tension ( $P_{aCO_2}$ ), and acid-base status were intermittently measured and corrected if necessary. Body temperature was monitored with a rectal probe and maintained at 36°–38°C by a heat lamp. Surgical anesthesia was maintained with repetitive doses of pentobarbital. The administration of repeat doses of pentobarbital was based on cardiovascular signs of inadequate anesthesia.

Rats were excluded from the experiment if they were hemodynamically unstable (mean arterial blood pressure < 80 mmHg) at the beginning of the experiments or if their blood gas status throughout the experiment was not within the defined predefined ranges ( $P_{aO_2} > 100$  mmHg; pH 7.36–7.44;  $P_{aCO_2} = 36$ –44 mmHg; base excess  $-2 \pm 2$  mEq).

### *Neuromuscular Function Test*

Neuromuscular transmission and function were monitored by evoked mechanomyography using a peripheral nerve stimulator (NS252; Fisher & Paykel Health Care, Irvine, CA) along with a Grass Force transducer and software (Grass Instruments, Quincy, MA). For this purpose, rats were placed in the dorsal recumbent position. The tendon of the insertion of tibialis cranialis muscle was surgically exposed on each side and individually attached to separate Grass FT03 force displacement transducer (Grass Instruments). Both sciatic nerves were exposed at its exit from the lumbosacral plexus at the thigh and tied with ligatures. Distal to the ligatures, stimulation electrodes were attached for nerve-mediated stimulation of the tibialis cranialis. The knees were stabilized rigidly with a clamp. A baseline tension of approximately 50 g was applied to the tendon of each tibialis cranialis muscle, which yielded optimal evoked tensions. The nerve-evoked tension of the respective tibialis muscle was recorded *via* a Grass P122 amplifier and displayed using the Grass Polyview Software (Grass Instruments).

Initially, during supramaximal stimuli, baseline twitch tension was established. The baseline mechanomyographic responses were stabilized over a period of 10

min using the train-of-four (TOF) stimulation pattern, every 20 s. The evoked muscle tension developed during TOF stimulation was recorded at the end of the 10-min period. This was followed by tetanic stimulation at 50 Hz for 5 s to assess the maximal tetanic muscle tension and the muscle fade associated with this stimulus. After an interval of 30 min with ongoing TOF stimulation every 20 s, to allow muscle recovery from the effects of the preceding tetanus, the potency of atracurium, a nondepolarizing muscle relaxant, was tested on both sides by the cumulative dose-response method. Bolus doses of atracurium were given intravenously in increments of 0.1–0.4 mg/kg until the first twitch height (T1) of the TOF was below 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, the twitch was allowed to recover to baseline values.

After complete recovery of T1 (> 95% recovery of twitch height), a continuous infusion of atracurium was started, and the infusion rate was adjusted to achieve a constant 50% neuromuscular block on the botulinum toxin-injected or saline-injected side. After 10 min of stable 50% twitch depression on the injected side at that stable infusion rate, steady state conditions were assumed to be present. At this point, the twitch height on the noninjected side was also noted during the steady state 50% twitch suppression on the injected side. One milliliter of heparinized blood was withdrawn for later determination of plasma concentrations of atracurium. The blood was immediately transferred to an Eppendorf tube containing 20  $\mu$ l H<sub>2</sub>SO<sub>4</sub>, 1 M, and centrifuged (3,500 rpm, 10 min, 4°C). The plasma was collected, and 0.2-ml portions were aliquoted into Eppendorf tubes containing 0.8 ml H<sub>2</sub>SO<sub>4</sub>, 15 mM. The samples were immediately frozen at –80°C. After blood sampling, tibialis cranialis, gastrocnemius, and soleus muscles on both sides were harvested, weighed, snap frozen on dry ice, and stored at –80°C for later biochemical analysis of nAChR concentration.

#### *Acetylcholine Receptor and Atracurium Assay*

The nAChR protein expression in the tibialis cranialis muscles was assayed using the <sup>125</sup>I- $\alpha$ -bungarotoxin binding assay, as previously described,<sup>24</sup> and expressed as femtomole nAChRs 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). The plasma concentration of atracurium was determined by high-performance liquid chromatography as described previously.<sup>25</sup>

#### *Data and Statistical Analyses*

All values are expressed as mean  $\pm$  SEM. Assuming that the twitch response or relation of the neuromuscular

block to the dose of atracurium is 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 every rat on each leg. These linear relations are typically characterized by slope (Hill coefficient) and intercept. Individual effective doses (EDs) were calculated by simple retransformation into linear scale. Values were compared by two-way repeated-measurement analyses of variance, using dose (between groups) and leg (intragroup and intergroup) as independent factors. *Post hoc* testing was performed with paired or unpaired *t* tests ( $P < 0.05$ ).

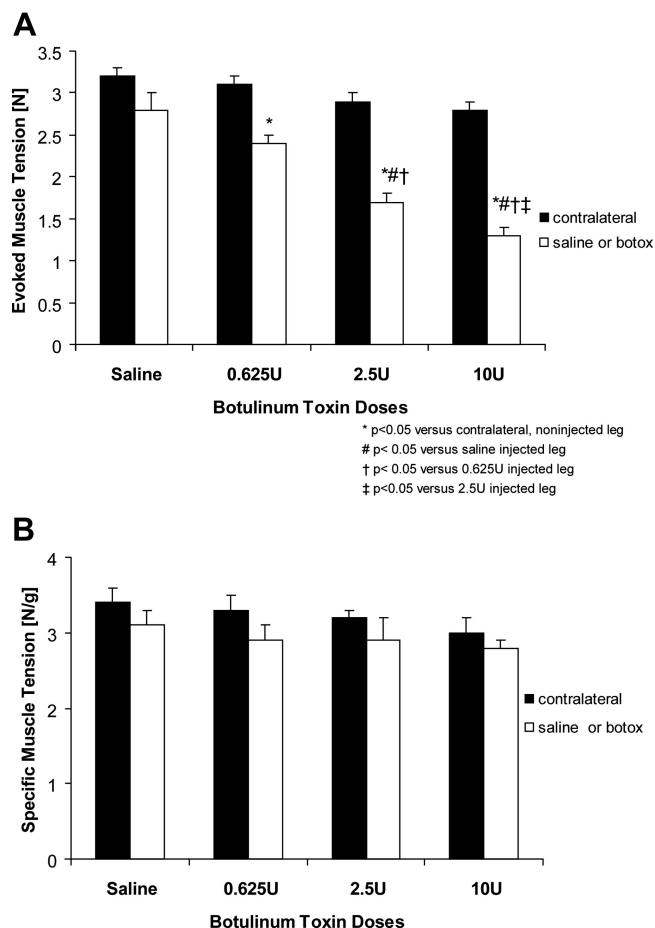
## Results

### *Model Stability*

All injected animals survived for 128 days. Originally, 12 rats were injected in each group. Hemodynamic and metabolic variables were monitored throughout the functional and pharmacologic studies. Nine rats in total were excluded from the experiment because of hemodynamically instability (mean arterial blood pressure < 80 mmHg) at the beginning of the experiments or because their blood gas status throughout the experiment was not within the defined predefined ranges (PaO<sub>2</sub> > 100 mmHg; pH 7.36–7.44; PaCO<sub>2</sub> = 36–44 mmHg; base excess  $-2 \pm 2$  mEq) ( $n = 9$ ). Therefore, the final statistical analyses included  $n = 9$  in the saline group,  $n = 11$  in the 0.652-U group,  $n = 10$  in the 2.5-U group, and  $n = 9$  in the 10-U group. In these animals, hemodynamic and metabolic values were not different between groups. During the cumulative dose-response, the mean Pco<sub>2</sub> was  $37.8 \pm 1.3$ , arterial blood pH ranged between 7.36 and 7.44, and base excess was  $-0.5 \pm 0.6$ .

### *Muscle Contractility and Fatigability*

At 128 days after injection of toxin, there were no differences in the tibialis muscle tensions between the saline-injected and contralateral side, and also the side contralateral to botulinum toxin injection. Relative to these muscles, there was a significant decrease in evoked muscle tension on the botulinum toxin-injected side in a dose-dependent manner. Animals injected with 0.625, 2.5, or 10 U showed a significant ( $P < 0.05$ ) decrease in evoked single twitch tension (T1 of TOF) ranging from 77.4% to 46.4%, respectively, compared with the contralateral (noninjected) side. The muscle tension was also significantly ( $P < 0.05$ ) reduced to 60.7% (2.5 U) and 46.4% (10 U) when compared with the saline-injected controls (fig. 1A). Although the animals injected with 0.625 U had lower twitch tensions compared with the contralateral side, the tension in limb injected with 0.625 U was not different compared with the saline-injected control. The specific muscle tensions (twitch tension per mg wet tibialis muscle mass) were not dif-

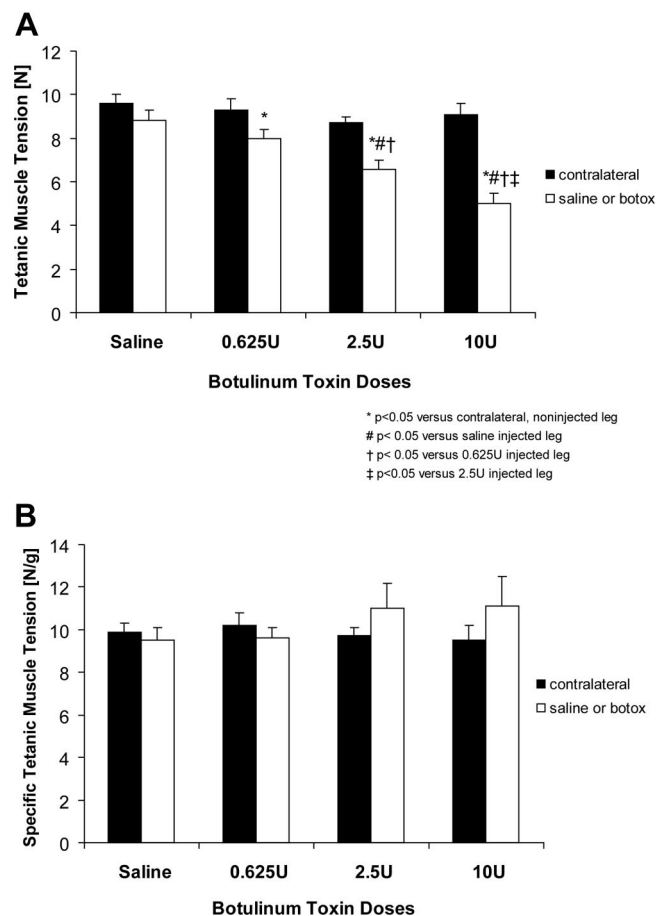


**Fig. 1.** Evoked muscle tension (N) and specific muscle tension (N/g) in the tibialis muscle. At 128 days after botulinum toxin or saline injection, the tension-generating capacity on the injected side, as well as the contralateral side, which received no injection, was measured. Separate animals with saline injection on one side and the contralateral naive side served as controls. (A) Evoked muscle tension was significantly decreased in a dose-dependent manner on the botulinum toxin-injected side relative to the contralateral side. Muscle contraction was also significantly reduced in the 2.5-U and 10-U groups when compared with saline-injected controls. (B) Specific muscle tension (tension per mg wet tibialis muscle mass) generated was not different between the experimental and control groups.

ferent between the botulinum toxin-injected side, the contralateral (noninjected) side, and the saline-injected controls (fig. 1B). Response to tetanic stimulation and the calculated specific tetanic tensions followed a pattern, similar to that of single twitch tension in that the absolute tetanic tensions were decreased on the toxin side but not the specific tetanic tensions (figs. 2A and B). Fade after TOF or tetanus was not different between groups.

#### *Atracurium Pharmacodynamics*

The ED<sub>10</sub> of atracurium was significantly reduced in the leg receiving 10 U botulinum toxin (table 1). No differences, however, were observed in the ED<sub>50</sub> values between the experimental and the contralateral (noninjected) side or the saline-injected and contralateral mus-



**Fig. 2.** Tetanic muscle tension (N) and specific tetanic muscle tension (N/g) in the tibialis muscle. At 128 days after botulinum toxin or saline injection, the tetanic tension-generating capacity on the injected side, as well as in contralateral side, which received no injection, was measured. Separate animals with saline injection on one side and the contralateral naive side served as controls. (A) Tetanic muscle tension was significantly decreased in a dose-dependent manner on the botulinum toxin-injected side relative to the contralateral side. Muscle contraction was also significantly reduced in the 2.5-U and 10-U groups when compared with saline-injected controls. (B) Specific tetanic muscle tension (tension per mg wet tibialis muscle mass) generated was not different between the experimental and control groups.

cles. The plasma concentration of atracurium to achieve steady state 50% neuromuscular paralysis on the toxin injected side was significantly lower in the highest botulinum toxin dose group (10 U) compared with the saline-injected group or the medium-dose (2.5-U) and low-dose (0.625-U) botulinum toxin groups (table 1). The plasma atracurium concentrations to achieve 50% neuromuscular paralysis on the injected side in the medium- and small-dose toxin groups were similar to that of controls. (The infusion rate was titrated to achieve a 50% neuromuscular block on the injected side.) The twitch suppression obtained on the contralateral side was similar to the injected side at all times, except in the 10-U group. The measured block achieved on the contralateral (noninjected) side was significantly smaller in the highest toxin dose (10-U) group (table 1). In contrast to

**Table 1. Atracurium Pharmacodynamics, Steady State**

		Saline	0.625 U	2.5 U	10 U
ED <sub>10</sub> of atracurium, mg/kg	Contralateral side	0.21 ± 0.05	0.25 ± 0.06	0.26 ± 0.04	0.30 ± 0.04
	Injected side	0.24 ± 0.05	0.28 ± 0.04	0.21 ± 0.03	0.16 ± 0.05*†‡
ED <sub>50</sub> of atracurium, mg/kg	Contralateral side	0.36 ± 0.07	0.42 ± 0.07	0.44 ± 0.04	0.50 ± 0.07
	Injected side	0.40 ± 0.06	0.48 ± 0.05	0.46 ± 0.04	0.41 ± 0.06
ED <sub>90</sub> of atracurium, mg/kg	Contralateral side	0.68 ± 0.07	0.71 ± 0.07	0.74 ± 0.06	0.85 ± 0.09†‡
	Injected side	0.69 ± 0.09	0.83 ± 0.09	1.00 ± 0.10	1.04 ± 0.09*†‡
Slope of dose-response curve	Contralateral side	3.9 ± 0.4	4.3 ± 0.4	4.2 ± 0.2	4.3 ± 0.2
	Injected side	4.2 ± 0.4	4.1 ± 0.4	2.9 ± 0.2*†‡	2.6 ± 0.3*†‡
Atracurium concentration, μg/ml, at 50% paralysis	Injected side	2.2 ± 0.3	2.1 ± 0.3	2.2 ± 0.2	1.2 ± 0.2†‡§
Twitch depression, %, during 50% paralysis on injected side	Contralateral side	45 ± 5	60 ± 4†	47 ± 5	16 ± 4†‡§

\*  $P < 0.05$  vs. contralateral, noninjected leg. †  $P < 0.05$  vs. saline-injected leg. ‡  $P < 0.05$  vs. 0.625 U-injected leg. §  $P < 0.05$  vs. 2.5 U-injected leg.

ED<sub>10</sub> or ED<sub>50</sub>, the ED<sub>90</sub> in the 10-U group was significantly increased on both the botulinum toxin and the contralateral noninjected side relative to the saline-injected and the contralateral leg. The trend for increased ED<sub>90</sub> on the 2.5 U-injected side did not reach statistical significance ( $P > 0.05$ ). Consistently, the slopes of the dose-response curve were significantly reduced in the groups receiving botulinum toxin doses of 2.5 and 10 U compared with the contralateral (noninjected) leg or the saline-injected leg (table 1).

#### Muscle Mass

There was a significant ( $P < 0.05$ ) dose-dependent decline in the tibialis cranialis muscle mass to 80.0% (0.625 U), 66.6% (2.5 U), and 50.0% (10 U) on the injected side compared with the contralateral (noninjected) leg. The tibialis cranialis muscle on botulinum toxin-injected side also weighed 88.8% (0.625 U), 66.6% (2.5 U), and 55.5% (10 U) of the saline-injected controls (table 2). Muscle mass of the adjacent gastrocnemius muscle was significantly ( $P < 0.05$ ) reduced in the 10-U group when compared with the contralateral (noninjected) leg and the 0.625 U- and 2.5 U-injected legs. There were no differences when compared with the saline-injected leg. Muscle mass of the adjacent soleus muscle was also significantly ( $P < 0.05$ ) decreased in the high-dose group compared with the contralateral side, the saline-injected leg, and the 0.625 U- and 2.5 U-injected legs (table 2).

**Table 2. Muscle Mass, in Grams**

		Saline	0.625 U	2.5 U	10 U
Tibialis muscle mass	Contralateral side	1.0 ± 0.0	1.0 ± 0.1	0.9 ± 0.0	1.0 ± 0.1
	Injected side	0.9 ± 0.0	0.8 ± 0.0*†	0.6 ± 0.0*†‡	0.5 ± 0.0*†‡§
Gastrocnemius muscle mass	Contralateral side	2.4 ± 0.1	2.4 ± 0.1	2.4 ± 0.1	2.3 ± 0.1
	Injected side	2.1 ± 0.3	2.4 ± 0.1	2.2 ± 0.1*	1.6 ± 0.2*†§
Soleus muscle mass	Contralateral side	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
	Injected side	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0*†‡§

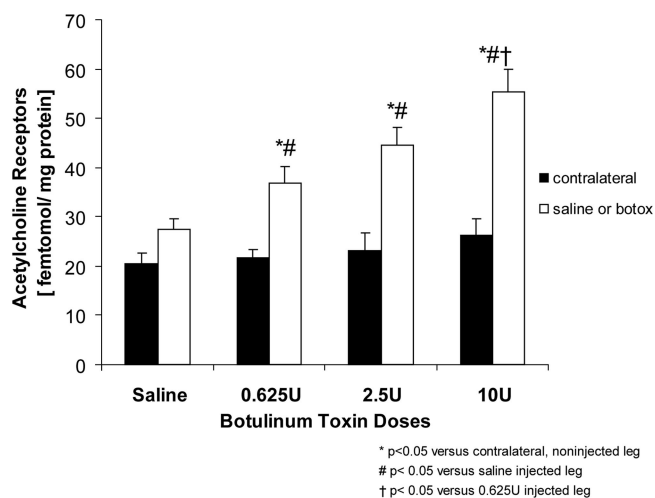
\*  $P < 0.05$  vs. contralateral, noninjected leg. †  $P < 0.05$  vs. saline-injected leg. ‡  $P < 0.05$  vs. 0.625 U-injected leg. §  $P < 0.05$  vs. 2.5 U-injected leg.

#### Acetylcholine Receptor Expression

The concentrations of membrane nAChRs in the tibialis anterior muscle were significantly ( $P < 0.05$ ) increased compared with that of contralateral (noninjected) leg and to that of the saline-injected controls (fig. 3).

#### Discussion

Our study documents that a single injection of the toxin can have long-lasting effects, even at 128 days after the initial injury. Despite lack of obvious anatomical denervation, our study confirms that the toxin can produce dose-dependent muscle weakness and a denervation-like state, evidenced as up-regulation of nAChRs. This observation is consistent with previous observations of the short-term neuromuscular effects of botulinum toxin.<sup>22,23</sup> The absence of complete anatomical denervation was electrophysiologically confirmed by the presence of nerve-evoked muscle contraction in the tibialis anterior muscle. Contrary to expectations, a normal sensitivity to the neuromuscular effect of the nondepolarizing muscle relaxant, atracurium was observed in the 0.625-U and 2.5-U toxin groups, despite up-regulation of nAChRs. Another novel and unexpected finding was that the highest dose of toxin (10 U) had a variable response depending on the ED value examined; ED<sub>10</sub> of atracurium was smaller, ED<sub>50</sub> was normal, and ED<sub>90</sub> was increased on the botulinum toxin-injected side relative to the saline-injected controls. An increased sensitivity to atracurium, evidenced as a decrease in plasma concen-



**Fig. 3.** Acetylcholine receptor expression (nAChR; fmol/mg protein) in the tibialis muscle. At 128 days after botulinum toxin or saline injection, nAChR expression on the injected side, as well as on contralateral side, which received no injection, was measured. Separate animals with saline injection on one side and the contralateral naive side served as controls. The concentrations of membrane nAChRs in the tibialis cranialis muscle were significantly increased compared with that of the contralateral leg and to that of the saline-injected controls. The number of nAChRs was significantly increased in the 10-U group when compared with the 0.625-U group. There were no differences in nAChRs expression in the 2.5-U compared with the 0.625-U group and the 10-U group compared with the 2.5-U group.

tration requirement for a 50% paralysis, was also observed in the highest toxin (10-U) group, regardless of the profound up-regulation of nAChRs. The expression of nAChRs on the contralateral, noninjected side was similar to controls, suggesting that injection of the toxin does not have distant effects. Muscle mass of soleus and gastrocnemius on the ipsilateral side to botulinum toxin was decreased after injection of 10 U, suggesting spread to immediate local areas.

It is well known that botulinum toxin has almost exclusive effects at the nerve terminal, preventing the release of acetylcholine. Therefore, after botulinum toxin poisoning, despite normal nerve conduction up to the nerve terminal, release of acetylcholine does not occur.<sup>12,13</sup> The toxin, taken up by the acetylcholine vesicles, cleaves the SNARE proteins (SNAP-25, synaptobrevin, and syntaxin), preventing the assembly of the fusion complex and thus blocking the release of acetylcholine.<sup>26,27</sup> Recovery from the effects of the neurotoxin is partly due to axonal sprouting around toxin-blocked receptors and the formation of new motor endplates. Rate, frequency, and success of nerve sprouting seem to play an important role in determining recovery.<sup>28</sup> So far, it has not been determined how the toxin is metabolized or otherwise eliminated from the nerve ending. The effects of botulinum toxin are, therefore, similar to the effects of sodium channel blocker, tetrodotoxin, in that both prevent the release of acetylcholine at the nerve terminal leading to paresis.<sup>29,30</sup> An unexpected finding was that the botulinum toxin group did not show any

fade during TOF or tetanus. Tetrodotoxin-induced decreased acetylcholine release leads to increased quantal content and prolongation of endplate current increasing synaptic strength in that muscle, most likely due to up-regulated nAChRs.<sup>29,30</sup> It is tempting to speculate that a similar mechanism was operative during botulinum toxin-mediated paresis; an increase in synaptic strength, due to up-regulated nAChRs, may have prevented the potential for fade during botulinum toxin-induced paresis.

Although the toxin has no direct effect on the muscle, loss of muscle mass or muscle wasting was observed even 128 days after toxin exposure. This observation is consistent with the results of Billiante *et al.*,<sup>21</sup> who observed long-lasting electromyographic and histologically atrophic changes up to 144 days after injection of the toxin. In our study, despite the presence of nerve-mediated contraction, muscle tension did not return to normal levels at 128 days. The muscle weakness or decreased tension-generating capacity was most likely due to loss of muscle mass because the specific single twitch and specific tetanic tensions (muscle tension per gram muscle weight) were not different between the experimental group and controls. At the highest dose of toxin, the effects could be seen even in the immediate vicinity beyond the tibialis, where the muscle mass was decreased in the soleus and gastrocnemius on the same side, but not at distant sides. The ED<sub>90</sub> was, however, increased on the contralateral, noninjected side after the highest dose of toxin. This finding is difficult to explain, particularly because no changes in nAChRs or muscle mass were seen on the contralateral side. Nevertheless, anatomical denervation of one leg is known to have distant effects such as the diaphragm.<sup>31</sup>

Classic theory of receptor pharmacology suggests that in the presence of increased receptor number (up-regulated nAChRs), the antagonist concentration or dose requirement to achieve a given effect (*e.g.*, paralysis) by a competitive antagonist (*e.g.*, atracurium) is increased while there is hypersensitivity to agonists such as succinylcholine.<sup>32-33</sup> Clinical reports do confirm that patients with botulinum toxin infection demonstrate a hyperkalemic response to succinylcholine.<sup>7,8</sup> In the current study, however, a trimodal response to the neuromuscular effects of the nondepolarizing muscle relaxant, atracurium, was observed. A leftward shift in the ED<sub>10</sub>, a normal ED<sub>50</sub>, and a rightward shift in the ED<sub>90</sub> were observed for atracurium. The unaltered ED<sub>50</sub> and a higher ED for a more profound block (*e.g.*, ED<sub>95</sub>) has been observed previously during partial denervation,<sup>31</sup> another condition with up-regulation and altered isoform expression.

This study did not quantitate the altered isoform expression. Even if one quantitated isoform expression by immunohistochemistry, the contribution of new receptor isoforms to neurotransmission would be difficult to

assess. That a denervation state was indeed produced by the toxin was confirmed by the up-regulation of nAChRs. The possible reasons for the trimodal ( $ED_{10}$ ,  $ED_{50}$  and  $ED_{90}$ ) responses to atracurium are as follows: After botulinum toxin-induced denervation/immobilization, there is *de novo* expression of fetal forms of receptors. These new isoforms are expressed not only at the extra-junctional areas, but also perijunctionally and more importantly even junctionally, contributing to neurotransmission. Therefore, the mature receptors decrease in number and fetal isoforms increase in number at the junctional area. The increased sensitivity to atracurium ( $ED_{10}$ ) is probably related to inhibition by atracurium of the mature receptors that are decreased in number, resembling a myasthenic state. The decreased plasma concentration requirement for atracurium for 50% paralysis may reflect a continuum of the increased sensitivity of mature receptors. The unaltered  $ED_{50}$  is related to the interaction of atracurium with both mature and fetal isoform of receptors at midpoint. The discrepancy between  $ED_{50}$  and plasma concentration of atracurium to produce 50% paralysis may be due to hysteresis between dose, blood level, effect site concentration, and the lag-time for interaction of atracurium with the receptor during the cumulative dose-response studies. The fetal form of receptors consisting of this  $\gamma$  subunit and the  $\alpha_7$  subunit containing AChRs have decreased affinity for (resistance to) nondepolarizing muscle relaxants.<sup>31-35</sup> Therefore, blocking these remaining fetal nAChRs probably required higher doses. This could explain the higher  $ED_{90}$  and the flatter slope. The resistance to atracurium (increased  $ED_{90}$ ) may also be related to inactivity-induced increased synaptic strength, which is related to increased quantal content, prolonged endplate current, and increased nAChRs number.<sup>29,30</sup>

Healthy mammalian subjects typically present a slope of 3.5-5.6, irrespective of the neuromuscular blocking agent<sup>36</sup> and species. The slopes of approximately 4 in all control and contralateral legs and in the low dose (0.625 U)-injected leg are therefore consistent with previous observations in normals.<sup>36</sup> After injection of 2.5 or 10 U of toxin, the Hill coefficient ranged between 2.6 and 2.9 (table 1). These values indicate a flatter dose-response curve; *i.e.*, in these experimental groups, the affinity between nAChRs and atracurium was decreased. If the increased number of receptors consisted of the same isoform, one would expect a parallel rightward shift. Therefore, the flatter line also suggests an altered isoform expression, most likely due to fetal isoforms.

The clinical implications of this study are as follows: Typically, infection and immobilization as seen in the intensive care unit leads to up-regulation of nAChRs with resistance to nondepolarizing muscle relaxants<sup>31-35</sup> and increased sensitivity to succinylcholine with hyperkalemia.<sup>34,37,38</sup> Botulinum toxin-mediated up-regulation of nAChRs can lead to hyperkalemia with succinylcholine

as reported in humans.<sup>7</sup> Based on our study, it is reasonable to state that dose requirements for nondepolarizing muscle relaxants would be variable after botulinum toxin infection, depending on the neuromuscular block that is required. In some instances, increased sensitivity may be seen in the affected muscles. A recent case report<sup>39</sup> confirms the increased sensitivity to a nondepolarizer in a muscle (orbicularis oculi) after cosmetic botulinum toxin injections compared with adductor pollicis muscle. Conversely, resistance to nondepolarizers may also be seen as indicated by the increased  $ED_{90}$  in our study. Furthermore, recovery from neuromuscular dysfunction after botulinum toxin infection may take several months and would be dependent on the severity of the infection. Human botulism immune globulin, which neutralizes the toxin, is now available.<sup>11</sup> Whether treatment with this can attenuate neuromuscular changes needs further study.

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