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Short-term effects of botulinum toxin on the lateral rectus muscle of the cat

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Abstract Botulinum toxin type A (BTX) is often used as an alternative to surgery for the treatment of strabismus and many other motor or cosmetic problems. Although numerous studies established BTX as a powerful transmission-blocking agent at the neuromuscular junction, no evaluation of extraocular muscle (EOM) contractile properties after administration of BTX exists. Some anatomical studies on EOM fiber types suggested a long-term preferential effect of BTX on orbital layer, singly innervated muscle fibers. In this study, we examined the short-term effects of BTX on the contractile properties of normal lateral rectus muscle to determine the functional effect of BTX on muscle-force output over time. Measurements of muscle tension and the corresponding EMG evoked by stimulation of nerve VI were made hourly for up to 18 h following BTX administration. An intramuscular BTX injection of 2 U caused a dramatic decrease in maximum twitch and tetanic tension of the muscle in response to different frequencies of stimulation. This suppression developed gradually over time, with a concomitant reduction of EMG amplitude. No significant changes in muscle-speed-related characteristics (e.g., twitch contraction time, fusion frequency) were found. The results suggest a functional effect of BTX on all muscle fiber types, although, with the dose used, we did not observe complete muscle paralysis within the time of recording. The time course of muscle tension suppression by BTX also was frequency dependent, with the lower stimulation frequencies being more affected, suggesting

that implementation of higher frequencies could still produce adequate eye movements.

Keywords Extraocular muscle · Abducens · Eye movements · Oculomotor system · Strabismus

Introduction

Since its early experimental use on primate extraocular muscles in 1973 (Scott et al. 1973), the intramuscular injection of botulinum toxin type A (BTX) has grown in popularity as an alternative to surgery in the treatment of strabismus. Good results have been shown in achieving stereopsis in adults (Elston et al. 1985; Holmes et al. 2000; Scott 1980) and children (McNeer et al. 1997; Scott et al. 1990; Spencer et al. 1997; Tejedor and Rodriguez 1998; Tejedor and Rodriguez 1999; Tejedor and Rodriguez 2001). BTX also has been used as an effective therapeutic tool for many other movement disorders (Jankovic 1994) and is under consideration for others (Brin et al. 1993). The list of its rehabilitative and cosmetic applications is continually increasing.

Botulinum is a product mainly of the bacteria *Clostridium botulinum*. The clinically valuable attributes of BTX are its specificity for motor nerve terminals and its relatively long duration of action. It has been found that BTX acts on nerve endings to inhibit spontaneous and evoked mediator release (Habermann and Dreyer 1986; Simpson 1981). At the neuromuscular junction, the toxin binds preferentially to the motor nerve endings, followed by internalization by means of receptor-mediated endocytosis. Then BTX acts locally at motor nerve endings to irreversibly block acetylcholine release, thus producing flaccid paralysis or paresis (Blasi et al. 1993).

The result of botulinum-toxin-blocking neuromuscular transmission (Burgen et al. 1949) is a weakening of muscle and a reduction in overactivity, as observed in the clinic. While treatment with botulinum toxin (usually type A) is well established in ophthalmologic disorders, changes in muscle contractile properties and muscle fiber

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composition, e.g., myosin-heavy-chain expression (McLoon et al. 1999) following its local injection have not yet been evaluated. In this study, we begin that evaluation by examining the short-term effects of BTX on contractile properties of the cat lateral rectus muscle. Previous clinical and experimental observations suggested that muscle-force production would be dramatically decreased.

We also focused on how fast, and what contractile changes (e.g., twitch contraction time) underlie, the force decrease. It has been reported that, within minutes, the proprioceptive discharge from muscle spindles of injected unguulate eye muscle decreases dramatically, but with a very high dose (20 times that used for strabismus patients) (Manni et al. 1989). Also, the EMG activity of both medial and lateral rectus muscles injected with BTX in adult strabismus patients was stated to be suppressed the instant the fluid volume entered the belly of the muscle, although the degree of EMG suppression was neither illustrated nor specified (Dengis et al. 1998). These findings suggest an almost immediate initiation of the decrease in muscle-force production, with further decrease over time. Clinical assessment of BTX-treated strabismus patients is usually done 1–2 days following injection, when the effect of BTX on muscle activity probably has stabilized.

Materials and methods

Surgical preparation

All procedures and protocols for animal care and use were approved by Virginia Commonwealth University's Institutional Animal Care and Use Committee. Four adult cats (2.5–3.5 kg) were used. The animals were initially anesthetized with sodium pentobarbital (45 mg/kg, IP). Additional doses of sodium pentobarbital (6.5 mg) were provided IV during the experiment, as needed, to maintain deep anesthesia, judged by the absence of a blink reflex and withdrawal to paw pinch. A tracheostomy was performed and end-tidal CO₂, respiratory rate, heart rate, and arterial blood oxygen saturation were continuously monitored and maintained within a normal range. Body temperature was maintained at 37°C with a heating pad. After the experiment, the animals were euthanized with an overdose of sodium pentobarbital administered IV.

The animals were placed in a Kopf stereotaxic frame, and a midline incision from the forehead to the back of the neck was made. A frontal craniotomy was performed for placement of a bipolar electrode for abducens nerve stimulation in the brainstem (coordinates: posterior 4.5 mm, lateral 2 mm, depth 8.5 mm) (Snider and Niemer 1961). These coordinates were approached at an angle of 50° from the vertical in the sagittal plane. The temporalis muscle was retracted and the lateral bony orbit removed. The lateral rectus muscle and its tendon were exposed. Muscle temperature and moisture were maintained using mineral oil. All the above procedures have been described in detail elsewhere (Shall and Goldberg 1992).

Paralysis of the lateral rectus muscle was induced using BTX as supplied by Allergan Inc. (BOTOX). The toxin was injected into the belly of the muscle at a dosage of 2 U (0.08 ng). One unit of the drug corresponds to the calculated median intraperitoneal lethal dose (LD₅₀) in mice. The dose we used was within the manufacturer's recommended range for humans (up to 2.5 U are used to affect a change of less than 20 prism diopters in patients with horizontal strabismus). However, it should be noted that the cat eye

lateral rectus muscle contains about half the number of muscle fibers (≈15,000 versus ≈30,000) found in humans (Peachey 1971). The volume of BTX injection was 80 μl, which is well within the manufacturer's suggested volume range (0.05–0.15 ml). A volume control was done by injecting 80 μl 0.9% NaCl into a separate cat lateral rectus muscle.

Stimulation and recording procedures

Lateral rectus whole muscle contractile properties were examined in response to abducens nerve stimulation. The abducens nerve was stimulated with a stainless steel bipolar electrode (0.2 mm tip diameter and 2 mm between poles) with about 1 mm of uninsulated surface at the pole tips. Optimal electrode placement was assessed based on the maximal contractile muscle response to stimulation.

The nerve was stimulated in three paradigms: (1) rectangular pulses of 700–800 μA intensity and 0.1 ms duration were delivered to the nerve at 1 Hz, and whole muscle twitch responses were recorded; (2) pulse trains of 400–800 μA intensity and 200 ms duration with a constant frequency in the range 50–220 Hz (eight different frequencies) were used to evaluate tetanic muscle responses – the trains were delivered in 5 s intervals to assure a return to baseline, and; (3) tetanic pulse/step trains of 700 μA intensity, 200 ms duration and variable frequency (sequence of 350 Hz for 15 ms, 200 Hz for 10 ms, and 100 Hz for 175 ms) were used to simulate the pulse/step activity seen during normal saccades (Fuchs et al. 1988; Moreno-Lopez et al. 1994). All stimuli to the abducens nerve were produced by a programmable pulse generator (AMPI Master-8). Stimuli intensity was chosen to be supramaximal in order to activate the whole nerve. This was determined experimentally by the lack of further increase in muscle-force response as intensity was increased.

Lateral rectus muscle tension evoked in response to abducens nerve stimulation was measured with a strain gauge (Pixie model 8108, Endeveco) with linear characteristics in the range 1–100 g. The muscle was detached from the sclera and its tendon was sutured with 5–0 silk thread and attached to the strain gauge. The strain gauge carrier was positioned at a muscle length that resulted in maximal isometric twitch tension when stimulating the abducens nerve. It has been shown that the length of the lateral rectus muscle yielding a maximal twitch tension is the same as for maximal tetanic tension (Barmack et al. 1971). Since the globe was not removed, a sclera suture was used to pull the globe medially, and was then fixed to the stereotaxic frame. This was done in an attempt to isolate the lateral rectus muscle contractions from the simultaneous contractions of the four retractor bulbi muscle slips, which also are innervated by nerve VI (Crandall et al. 1981).

Lateral rectus muscle EMG activity was recorded using a bipolar electrode consisting of two flexible stainless steel wires (25 μm diameter) inserted into the muscle (Shall and Goldberg 1995).

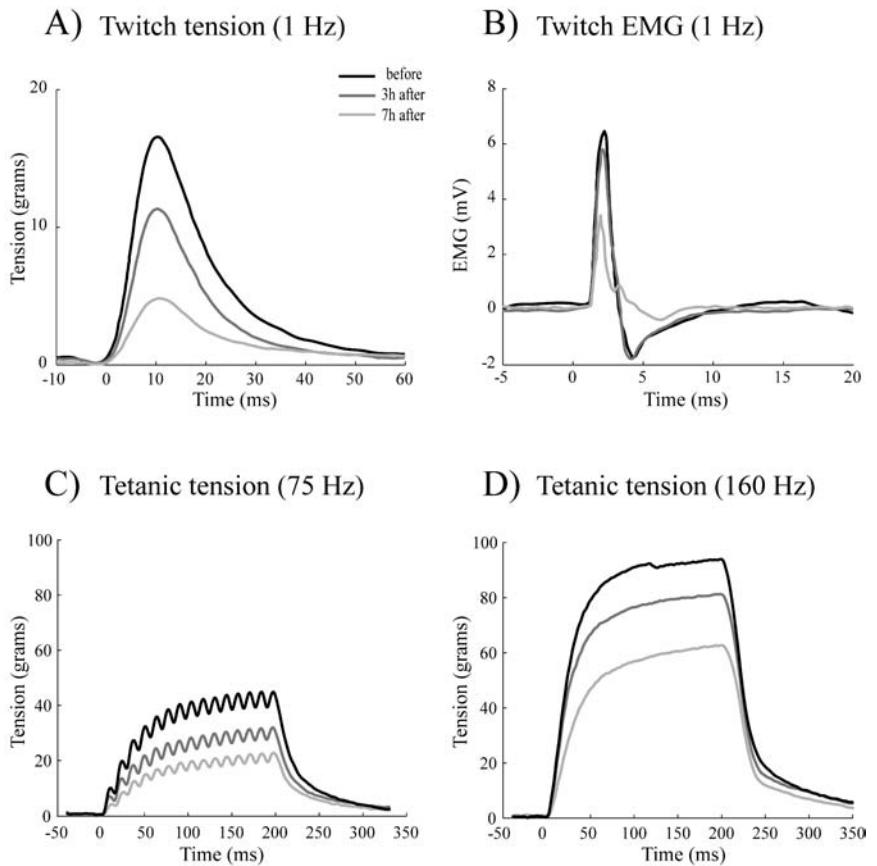
Data analysis

Muscle force and EMG responses in the different stimulation paradigms were monitored and recorded for off-line analysis in four cats before injection, immediately after the injection, and at every hour after that for 7 h. One animal was followed for up to 18 h after injection. At least ten individual twitch responses and three trials for each tetanic response, at each of the eight frequencies used, were recorded in all animals.

Maximum amplitude of the lateral rectus twitch response, as well as contraction time and half-decay time, were measured. Maximum amplitude of tension was measured also for tetanic responses. In the pulse/step paradigm, the amplitude of tension was measured for both pulse (peak) and step phase (at 150 ms after tension onset) of the response. Magnitude of the EMG was measured as the peak-to-peak amplitude.

Lateral rectus contractile characteristics were examined using analysis of variance (ANOVA) for the effect of BTX over time, the

Fig. 1 Samples of twitch and tetanic responses of lateral rectus muscle in one cat before and at the third and seventh hour after botulinum toxin type A administration. **A** Twitch tension; **B** twitch EMG; **C** tetanic tension at stimulation frequency of 75 Hz; and **D** tetanic tension at stimulation frequency of 160 Hz. (Legend in **A** applies also to **B**, **C**, and **D**.)



stimulation frequency used, and the subject tested. ANOVA results are presented with the F -value (degrees of freedom of the sample, degrees of freedom of the error) and the significance level P . A linear regression analysis was also performed. A probability level $\alpha=0.05$ was used in all statistical evaluations. Values presented in the text and in the figures are given as mean \pm SE.

Results

Muscle twitch response

Examples of twitch-tension recordings and the corresponding EMG, before and after injection, are illustrated in Fig. 1A and B, respectively.

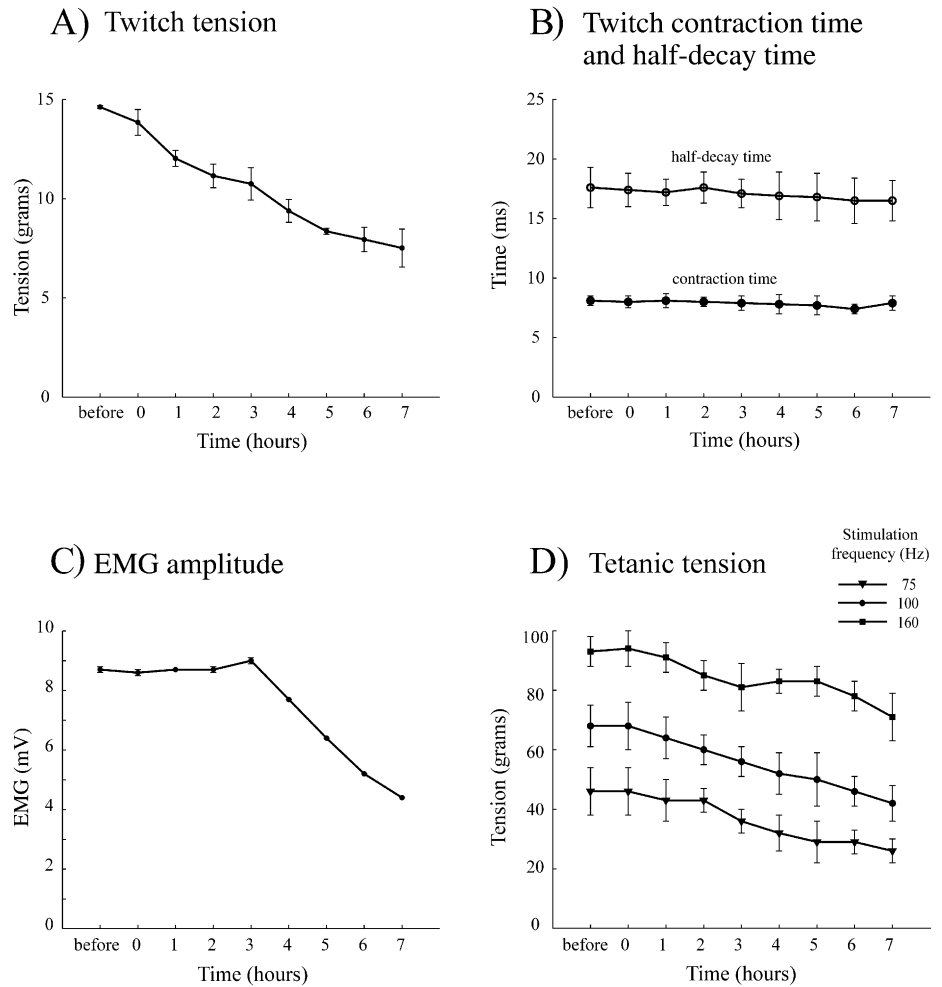
Maximum force produced in response to single-pulse stimulation was significantly affected by the BTX ($F_{(8,24)}=28$, $P<0.001$). Twitch tension gradually decreased over time from 14.6 ± 0.1 g before the application of BTX, to 7.5 ± 1.0 g at the seventh hour postinjection (Fig. 2A). The decrease in maximum twitch tension over time also was subject dependent (interaction: $F_{(24,252)}=67$, $P<0.001$). Although for each cat the twitch tension decreased after application of BTX, variability of decrease among the tested cats can be seen in Fig. 2A. Injecting lateral rectus muscles with a control volume caused a slight decrease in twitch tension (from 12.4 ± 0.1 g to 11.6 ± 0.1 g), similar to the twitch tension decrease recorded right after BTX injection (time 0 in Fig. 2A

compared to the tension before BTX injection). Further decrease in muscle tension over time was not found for the control injection.

Temporal characteristics of the twitch response, however, did not change after BTX application. Both contraction time and half-decay time of the twitch (Fig. 2B) remained the same during the hours following injection (contraction time: 8.1 ± 0.4 ms before injection and 7.9 ± 0.6 ms at the seventh hour postinjection, $F_{(8,26)}=0.14$, $P>0.99$; half-decay time: 17.6 ± 1.7 ms and 16.5 ± 1.7 ms, respectively $F_{(8,26)}=0.07$, $P>0.99$).

Amplitude of muscle EMG activity also decreased (Fig. 1B and Fig. 2C) concomitant with the decrease in tension from 8.7 ± 0.1 mV before injection to 4.4 ± 0.0 mV at the seventh hour postinjection ($F_{(7,79)}=791$, $P<0.001$). The observation that EMG amplitude, in contrast to twitch tension, showed minimal decline for 3 h after injection (Fig. 2A and C) might imply that larger diameter muscle fibers are the last to be affected by BTX. This is based on the assumption that large diameter fibers contribute most to EMG amplitude. However, 7 h after injection, both twitch tension and EMG amplitude were at about 50% of their initial values.

Fig. 2 Time course of **A** twitch tension, **B** twitch contraction time and half-decay time, **C** EMG peak-to-peak amplitude, and **D** tetanic tension responses to different stimulation frequencies after a single botulinum toxin type A (BTX) injection into cat lateral rectus muscle. The data on each graph is presented as mean \pm SE. Note: time 0 corresponds to recordings right after BTX injection



Muscle tetanic response

The effects of BTX, coupled with the frequency of stimulation on muscle responses, were studied (Fig. 1C and D; Fig. 2D). Recordings at different hours before and after injection were plotted for the 75 Hz stimulation frequency (Fig. 1C) and for 160 Hz (Fig. 1D).

As expected, increasing the stimulation frequency led to an increase in muscle-force production ($F_{(8,24)}=69$, $P<0.001$), and this relationship was preserved after the administration of BTX. The amplitude of tetanic responses for three frequencies used is illustrated over time in Fig. 2D. Application of BTX caused a gradual decrease in muscle force over time during tetanic stimulation, similarly to single pulse stimulation, for all frequencies used ($F_{(8,23)}=9.7$, $P<0.001$). However, an interaction between the effects of BTX and the frequency of stimulation was found ($F_{(64,181)}=1.5$, $P<0.05$). Tension at lower tetanic frequencies decreased to about half the initial one compared to higher frequencies, where tension decreased only by one fifth (e.g., 75 Hz and 160 Hz in Fig. 2D). We noted no changes in tetanic fusion frequency after injection, similar to the lack of change in twitch contraction time. Injection of a volume control into the

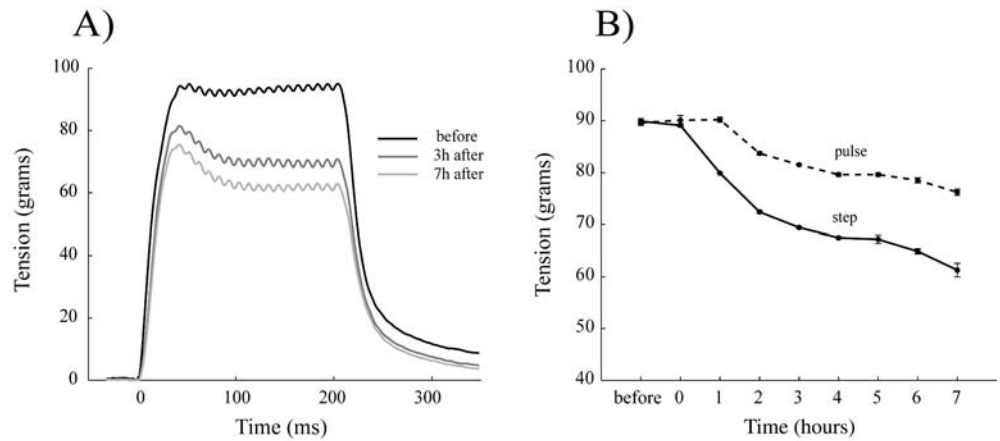
lateral rectus muscle showed no significant effect on tetanic tension. Indeed, even injection of BTX yielded no immediate decline in tetanic tension (compare tension at 0 ms and before injection in Fig. 2D).

Similar to twitch responses, the effects of both frequency and time on the amplitude of tetanic tension following injection depended on the cat tested (interaction between frequency and subject, $F_{(24,181)}=38$, $P<0.001$; interaction between time and subject, $F_{(23,181)}=25$, $P<0.001$). That is, there was some variability among the animals, as tetanic force declined after injection (Fig. 2D).

Pulse/step responses

Stimulating the nerve at 100 Hz (step) immediately following a 25 ms high frequency pulse showed little, if any, reduction in tension compared to pulse tension before administration of BTX, but a clear step phase became evident after injection (Fig. 3A). Decrease in both pulse and step amplitudes over time is illustrated in Fig. 3B. Within the hours after injection, both force amplitudes in response to pulse stimulation and during the 100-Hz step stimulation decreased significantly

Fig. 3 **A** Samples of pulse/step tension responses before and at the third and seventh hour after botulinum toxin type A (BTX) administration. Note the increasing difference between pulse and step force amplitudes after BTX administration. **B** Time course of pulse and step amplitudes after BTX administration. Pulse tension was measured at the peak and step tension was measured at 150 ms after tension onset



($F_{(8,36)}=375$, $P<0.001$). Step force, however, decreased at a faster rate, as seen in Fig. 3B (interaction: $F_{(8,36)}=27$, $P<0.001$).

Contractile responses 18 h after BTX administration

If the gradual decrease in time of muscle twitch tension was approximated with a straight line ($R^2=0.89$, $y=13.4-0.87x$, $P<0.001$), the regression analysis predicted a complete suppression of twitch force between the 15th and 16th hours postinjection. To check whether muscle force can be completely suppressed by BTX, muscle responses in one cat were recorded up to 18 h after injection. Results at the 18th hour showed that maximum twitch tension was greatly, but not completely, reduced (1.1 ± 0.0 g compared to the initial 14.8 ± 0.1 g before injection). EMG amplitude was also further decreased, being 0.8 ± 0.0 mV at the 18th hour postinjection. Tetanic tension, also greatly reduced, confirmed by the frequency dependence found at the seventh hour after administration. For example, for 50 Hz stimulation frequency, tetanic tension was ten times smaller at the 18th hour (3.7 ± 0.0 g compared to 35.4 ± 0.0 g before injection), while for the highest stimulation frequency used (220 Hz), decrease in tension was about three times (27.5 ± 0.0 g compared to 97.9 ± 0.0 g). Twitch contraction and half-decay time at the 18th hour were 8.6 ± 0.1 ms and 18.4 ± 0.1 ms compared to 8.5 ± 0.2 ms and 17.7 ± 0.2 ms, respectively, prior to injection.

Discussion

We found that the IM injection of BTX (2.0 U – a dose often used clinically) into the cat lateral rectus muscle greatly suppressed both twitch and tetanic force, as well as EMG amplitude. This suppression developed gradually over time. While the immediate force reduction in twitch-tension response noted right after administration may be attributed to the volume injected, the continuous decrease within the next hours evidently is due to toxin's effect on

muscle contractile properties. Although amplitude of the tension was decreased, as expected, twitch and tetanic speed-related characteristics (i.e., twitch contraction time, half-decay time, and tetanic fusion frequency) did not appear significantly altered.

BTX may affect all muscle fiber types

That we observed little change in twitch contraction time and fusion frequency over time may have interesting implications. Spencer and McNeer (1987) noted that singly innervated orbital fibers showed the most clear morphological changes after injection of BTX (10 U) into the medial rectus muscles of adult rhesus monkeys. In addition to this anatomical observation on singly innervated orbital fibers, these authors also assumed that BTX affected physiologically all muscle fiber types (Spencer and McNeer 1987). It should be mentioned that rhesus monkeys have about 10,000 medial rectus muscle fibers as opposed to about 28,000 in the adult human (Peachey 1971). Accordingly, 10 U of BTX might have a greater effect on the rhesus than on humans. That we observed little alteration in lateral rectus muscle-speed-related contractile characteristics over the first hours after administration may confirm that BTX functionally affects all extraocular muscle fiber types. Any preferential effects of BTX on a particular fiber type should lead to contraction time or fusion frequency changes. Indeed, somewhat higher doses of BTX completely paralyze the lateral rectus muscle in the cat (Moreno-Lopez et al. 1997). The lack of change in the temporal properties of BTX-treated extraocular muscles (EOM) also supports the notion that timing of the contraction is intrinsically determined by the myofiber proper. The response of fibers still innervated by the abducens nerve hours after administration suggests there is no short-term effect of BTX on these specific myofiber characteristics.

In contrast to this analysis, Stahl et al. (1998) report (for periorbital rather than intramuscular injection) some preferential BTX effect on singly innervated orbital fibers, since “static alignment” in their patients was

abnormal. The authors assume that singly innervated orbital fibers are preferentially used during this activity. However, saccades and other eye movements appeared relatively normal (Stahl et al. 1998). In addition, Dennehy et al. (1991) report that low doses (1–2.5 U) of BTX appear to spare multiply innervated fibers in the cat. This was judged by administration of succinylcholine, which evoked sustained contractions of the cat medial rectus muscle previously treated with BTX. However, most experiments were run at least 4 weeks after injection, and it has been shown that the cat lateral rectus muscle is essentially fully recovered by that time after administration of equivalent BTX doses (Moreno-Lopez et al. 1997). Dennehy et al. (1991) do report, in their discussion, that one animal showed similar sustained contractions 2 weeks after the administration of a very high dose (15 U), although this result was not included in their analysis. However, it should be made clear that postsynaptic action of succinylcholine may not be affected by BTX presynaptic chemodenervation of the muscle. Indeed, others have reported a total absence of eye movements after administration of similar high doses in the cat (Moreno-Lopez et al. 1997).

The approach used to record tension in this study involved the cat's isolated lateral rectus muscle with its tendon attached to a force transducer. Consistent with the active pulley hypothesis (Demer et al. 2000), it might be speculated that orbital layer fibers were excluded from our study, assuming that they insert on the pulley and do not impart force to the tendon. Our previous data regarding motor unit force measurement at the muscle tendon, and simultaneous and independent EMG recordings from the orbital and global layers of cat lateral rectus muscle (Shall and Goldberg 1995, Goldberg and Shall 1999), showed that 8 of 41 motor units studied were confined to the orbital layer, and 11 appeared to be split between global and orbital muscle layers. Thus, the recorded force at the tendon from orbital layer fibers may be a result of mechanisms for lateral force transmission within a muscle (see review of this important concept by Monti et al. 2001). Present data, therefore, does not exclude the orbital layer. It would be interesting to investigate EOM pulleys in BTX-treated strabismic patients, especially providing long-term structural changes in orbital muscle fibers (Spencer and McNeer 1987).

BTX effects are frequency dependent

Nerve VI stimulation at different frequencies confirmed the hypothesis that the effect of BTX on contractile properties of the muscle is frequency dependent. Lower frequencies were affected to a greater extent than higher ones over a 7 h period as well as over the longer period studied. At 220 Hz, we saw about 30% of our control tension after 18 h, while we saw only about 10% of our control value at 50 Hz. Moreno-Lopez et al. (1997) also recorded reliable but smaller eye movements with nerve stimulation at 250 Hz 1–7 days after injection.

With regard to this observed frequency effect, it has been noted that motoneuron firing patterns in the adult cat are essentially unchanged after BTX injection, even though eye movement amplitudes are reduced or abolished for an extended time and then recover (Moreno-Lopez et al. 1997). One might speculate, however, that those firing patterns would change after injection into the extraocular muscles of younger animals. There appears to be a trend, in the clinical literature we have cited in this paper, for BTX injection to have better long-term effects in young children (McNeer et al. 1997) as opposed to older children (Ing 1993) and adults. Therefore, higher frequencies of firing might be implemented to produce adequate eye movements in a permanently weakened but maturing muscle. It is also possible that the more substantial effects of BTX on EOM in young children might take place at the level of the developing muscle.

In addition, before injection, the pulse/step stimulation paradigm we used to approximate motoneuron firing during a saccade (Fuchs et al. 1988; Moreno-Lopez et al. 1994) revealed almost no drop in the evoked muscle force at 100 Hz following high frequency pulse (Fig. 3A, top trace). However, a clear drop in muscle force at 100 Hz was evident following injection (Fig. 3A). We of course expected absolute force level to decline following injection, even with the use of the pulse/step paradigm. However, use of the pulse/step paradigm further highlighted the importance of frequency when determining the effects of BTX.

Given the linear motoneuron discharge rate/eye position properties of the oculomotor system, frequency-dependent tension found to develop in BTX-treated cat lateral rectus muscle in this study also suggests that high frequency signals associated with saccades would be less affected than lower frequency signals associated with fixation near primary position and pursuit/vergence movements. Indeed, Stahl et al. (1998) showed that saccadic range and velocity in patients with transient diplopia following BTX treatment for craniofacial dystonia were within normal limits, whereas the static alignment of the eyes was abnormal.

Variability of responses to BTX injection

Although in all tested cats, muscle-force production was consistently suppressed by the injection of BTX, we saw some variability in the time course of force decline among individual subjects. This is in accordance with responsiveness of patients treated in the clinic (i.e., not all patients respond identically to similar doses). In addition to inherent differences among subjects, this response variability simply may be due to variations in the manner of injection and the amount of toxin that actually reaches the muscle endplates.

BTX suppresses both muscle force and EMG activity

While we observed a gradual decrease in twitch and tetanic force with a concomitant EMG amplitude decline, we never saw a total absence of force or EMG response in the lateral rectus muscle. Although we took precautions to mechanically immobilize the globe and retractor bulbi muscle, the possibility remains that there may have been minimal involvement of retractor bulbi contraction force in our experiments. However, we found a clear but low amplitude EMG in the isolated lateral rectus muscle after 18 h. Similarly, Moreno-Lopez et al. (1997) observed small lateral eye movements with high frequency (250 Hz) stimulation of nerve VI (as well as an “almost undetectable” EMG) 1 day after injection at doses equivalent to ours in the cat. These observations appear to indicate that the cat lateral rectus is greatly, but not completely, paralyzed 18–24 h after injection with 2 U of BTX. This finding also supports the clinical practice of evaluating BTX-treated patients 1–2 days after injection.

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