Botulinum toxin paralysis of the orbicularis oculi muscle. Types and time course of alterations in muscle structure, physiology and lid kinematics

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Abstract. In chronically prepared guinea pigs, we investigated the time course of botulinum toxin A's (Bot A) effect on the blink reflex by monitoring lid movements and EMG activity prior to and after Bot A injection into the orbicularis oculi muscle (OOemg), or after nerve crush of the zygomatic nerve. We correlated these alterations with the morphological changes of the orbicularis oculi (lid-closing) muscles of the same animals. After Bot A treatment there was a profound reduction of OOemg activity and blink amplitudes as well as a slowing of maximum blink down-phase velocity. Blink up-phases, however, remained unchanged. Gradual recovery of OOemg magnitude and blink amplitude started around day 6; a functioning blink reflex appeared on day 21, and full recovery of blink amplitude occurred by day 42. Crushing the zygomatic branch of the facial nerve produced similar changes in blink parameters, but recovery was much more rapid (15 days) than for Bot A-treated guinea pigs. The morphological analysis demonstrated that Bot A produced a denervation-like atrophy in the orbicularis oculi. No fiber type-specific alterations were noted, and all muscle fiber types ultimately recovered, with no long-standing consequences of the transient denervation. Our findings support the notion that functional recovery was the result of preterminal and terminal axonal sprouting that subsequently re-establishes functional innervation. Moreover, differences between the present findings and those seen after injection of Bot A into the extraocular muscles strongly support the hypothesis that the composition in terms of muscle fiber type and the properties of the motor control system of a given muscle greatly influence both how the particular muscle responds to toxin injection, and how effective the toxin is in resolution of neuromuscular disorders that affect a particular muscle. The present findings were consistent with clinical observations that Bot A produces only temporary relief in patients with essential blepharospasm. It is likely that the efficacy of Bot A in treatment of blepharospasm could be improved by using agents that suppress terminal sprouting. The close correspondence of the changes in blink physiology between human patients and guinea pigs after Bot A treatment demonstrate that the guinea pig is an excellent model system for testing strategies to prolong the beneficial effects of Bot A treatment in relieving lid spasms in human subjects.

Key words: Botulinum toxin – Blepharospasm – Dystonia – Blink – Guinea pig

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Introduction

In mammals, blinking occurs through the interaction of four forces acting on the upper eyelid. First, the tonically active levator palpebrae superioris muscle raises the upper eyelid. Second, a sympathetically controlled smooth muscle, Mueller's muscle, supplies an additional, small upward force. Third, the normally quiescent orbicularis oculi muscle provides an active downward force during its transient periods of activation. Fourth, there is a constant, passive downward force imposed by the attachments and structure of the ligaments and muscles of the eyelid (Evinger et al. 1991b; Sibony et al. 1991 for reviews). To blink, the nervous system briefly inhibits the levator palpebrae superioris motoneurons, and then a transient activation of orbicularis oculi motoneurons, in concert with the passive downward force, rapidly lowers the upper lid. When the orbicularis oculi motoneurons cease discharging, the levator palpebrae superioris motoneurons resume their tonic activity, and the eyelid rises, returning to its preblink position. This pattern becomes exaggerated and disrupted in some diseases, e.g., hemifacial spasm, Meige's syndrome and essential blepharospasm. Spasmodic involuntary orbicularis oculi contractions, in some cases leading to complete eye closure characterize these syndromes. The preferred treatment for

these disorders is injection of botulinum toxin A (Bot A) into the eyelid (e.g., Kraft and Lang 1988; Dutton and Buckley 1988). Bot A acts as a presynaptic blocker of acetylcholine release and thereby produces transient paralysis of the tonically overactive or spastic muscle (Gundersen 1980; Simpson 1986; Schantz and Johnson 1992, for reviews).

While Bot A therapy has proven to be preferable to many other treatments for blepharospasm (Kraft and Lang 1988), including surgery and centrally acting pharmaceuticals, the toxin is not without limitations. Bot A treatment for facial spasms must be repeated every 2 to 3 months for patients to remain asymptomatic (Scott et al. 1985). The long-term effects of repeated injections are unknown. In contrast, the eye realignment produced in strabismus patients by Bot A injections may outlast the period of paralysis. This long-term effect appears to be a consequence of adaptive changes in extraocular muscle morphology or oculomotor control systems, or both. Comparison of the structural/functional consequences of Bot A usage in blepharospasm and strabismus has shown that the efficacy of the toxin is, at least in part, related to the type of disorder (spastic versus positional or tonic) and to the fiber type composition and properties of the motor control system of the treated muscle (Spencer and McNeer 1987; Porter et al. 1991; Capra et al. 1991).

Injection of Bot A into human patients with hemifacial spasm or Meige's syndrome produces particular changes in the kinematics of lid movements as well as an elimination or reduction in the severity of orbicularis oculi spasms. Bot A treatment causes a dramatic reduction in blink amplitude and decreases the maximum velocity of the lid movement for lid closure only (Manning et al. 1990). These distinctive characteristics allow unambiguous correlation between the effects of Bot A on nonprimate mammalian blinks and its effects on human lid motility.

The present study analyzed the structural and functional consequences of Bot A injection into the orbicularis oculi muscle of individual guinea pigs. The toxin caused a generalized and reversible atrophy of all fiber types in the orbicularis oculi that correlated well with observed changes in lid movement function. Moreover, the changes in lid kinematics were similar to those occurring in humans (Manning et al. 1990). Thus, it is possible to correlate alterations in the orbicularis oculi muscle with modifications of lid movements within the same animal and to use these data to predict the effect of various drug treatments on humans where muscle morphology is unobtainable. The attainment of a thorough understanding of the morphological/physiological adaptations of the orbicularis oculi and its control systems to the onset and termination of botulinum-induced paralysis suggests modifications of current Bot A therapy to increase the length of its action. Such modifications might lead to improvements in the clinical management of blepharospasm. A preliminary report of these findings has been published elsewhere (Horn et al. 1991).

Materials and methods

Reflex blinks were studied in seven male, albino, guinea pigs (300–1000 g). Experimental procedures were in accordance with all Federal, State, and University guidelines and received local IACUC approval.

Surgical procedures

Under general anesthesia (40 mg/kg ketamine, 0.4 mg/kg acepromazine and 6 mg/kg xylazine) and aseptic conditions, animals were prepared for chronic recording of the electromyogram of the orbicularis oculi muscle (OOemg) in the upper eyelid and stimulation of the supraorbital branch of the trigeminal nerve, as described previously (Evinger et al. 1993). The OOemg activity was recorded with two teflon-coated stainless steel wires (A-M Systems; 0.0045″ diameter), bared 1 mm, that were inserted into the lateral aspects of the upper eyelid. A silver wire placed on the skull served as a ground. A teflon nerve cuff containing a pair of stainless steel wires was placed around the supraorbital branch of the trigeminal nerve as it left the skull. All wires were led subcutaneously to the top of the head and soldered to the female end of a miniature connector that was embedded into a dental acrylic platform. The dental acrylic platform was anchored to the skull by 6–8 self-tapping stainless steel screws (I-72, 5/8″). Two sets of nuts were embedded into the platform to allow fixation of the head to a head holder during testing. Postoperatively, the guinea pigs received prophylactic antibiotics and analgesics as necessary. At least 4 days after surgery, when the guinea pigs were completely recovered, the animals participated in their first experiment.

Data collection and analysis

In each test session, we monitored upper eyelid position, lid velocity and OOemg activity. To accomplish these measurements, we wrapped the guinea pigs in a tightly fitting cloth and placed them into a plastic container matched to their body size. The head was restrained by bolting the dental acrylic platform to a fixed bar. The animals were placed in a magnetic field and the lid position was measured with the magnetic search coil technique (Robinson 1966; Becker and Fuchs 1988; Evinger et al. 1991b). A micro coil (Sokyomat, Switzerland, 1.8 or 2 mm diameter, 80 or 100 turns) was taped to the lower margin of the previously shaved upper eyelid. Because of its low weight (2 mg) the coil did not interfere with lid movements. After each test session, the output signal of the search coil was calibrated with the lid position signal by taping the coil to an artificial eye matched to the diameter of the guinea pig eye and rotating it through known angles.

The lid position (0–500 Hz band pass) and OOemg signals (300 Hz–5 kHz) were collected (at 5000 Hz with 12 bit precision; DT2801A, Data Translation) and stored on a personal computer for later off-line analysis. The computer displayed each trial with lid position, lid velocity and the rectified OOemg. With assistance from the computer, the investigator identified the beginning, end, maximum closure, and maximum up and down velocities of the blink as well as the beginning and end of OOemg activity. The computer then calculated amplitudes, latencies and durations of the blinks (see Evinger et al. 1991b for details).

Reflex blinks were evoked either by a gentle air puff directed towards the cornea and periorbital region, or by electrical stimulation of the supraorbital nerve (SO; see Evinger et al. 1993 for details). Each session consisted of 10 to 12 blocks of 10 trials, of which the first 6 to 8 blocks were air puff stimuli. The time between trials within a block was 18 ± 3 s. We changed the stimulus intensity with each block. The smallest intensity, measured at the source (see Manning and Evinger 1986 for details), was 7 psi and the largest was 16 psi (duration 30–50 ms). The next four blocks of trials used SO

stimulation to evoke blinks. We changed the stimulus intensity after every block. The electrical stimulus was 100 μs and intensity varied between 1 and 4 mA. If habituation occurred at the 18 ± 3 s intertrial interval, we lengthened the interval and recollected the data. This procedure of changing air puff and electrical stimulus intensity produced a full range of blink amplitudes.

Treatment procedures

In all seven guinea pigs, we collected normal blink data for at least 3 days, then six of the animals received a Bot A injection. As a control for changes in lid kinematics induced by the injection, we gave an injection of saline into the eyelid one week before botulinum treatment in one guinea pig. Testing was resumed 1–2 days later for all guinea pigs. Data were collected for 4, 12, 21 or 41 days, until death of the animal. We crushed the zygomatic branch of the facial nerve in the remaining guinea pig, which was then killed 15 days after the crush.

Botulinum toxin injection

Before each injection, the Bot A (Sigma) solution was freshly prepared from small aliquots of a stock solution (0.5 mg/0.5 ml) that was stored at −20°C and diluted in saline. Under general anesthesia, we injected doses of 0.036–0.35 ng/kg body weight in a total volume of 4–8 μl at two sites at the lateral margins of the left upper eyelid, using a 33-gauge hypodermic needle attached to a Hamilton syringe. One animal that received three consecutive injections, at day 0, day 5 and day 15, survived 21 days after the third injection. We injected one animal with the same volume of saline as a control. In no case did we observe any signs of general Bot A intoxication or discomfort after the injection.

Nerve crush

In one animal, we performed a nerve crush of the facial nerve branch innervating the orbicularis oculi on the left side. Under general anesthesia, and using aseptic conditions, we made a small incision between the eye and ear and exposed the zygomatic branch of the facial nerve that innervates the orbicularis oculi (Uemura-Sumi et al. 1986). The zygomatic branch was identified by electrical stimulation through hook electrodes resulting in selective movements of the eyelid. We crushed the zygomatic nerve distally to the junction with the main nerve trunk between the tips of a pair of forceps (Dumont No. 5) for 40 s.

Morphology

After survival times of 4, 12, 21, or 42 days, all guinea pigs were perfused with a solution containing 0.6% dextran followed by a fixative solution containing 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M phosphate buffer. The eyelids were then removed bilaterally and immersed for 4 h in a fixative solution containing 4% glutaraldehyde in phosphate buffer. Eyelids were cut into 1-mm thick slabs and then washed in phosphate buffer, postfixed in 1% osmium tetroxide, stained en bloc with uranyl acetate, dehydrated in methanols and propylene oxide, and embedded in epoxy resin. Semithin and ultrathin sections were cut with an ultramicrotome. Semithin sections were stained with *p*-phenylenediamine and examined with phase contrast microscopy. Ultrathin sections were picked up on 135-mesh grids, stained with uranyl acetate and lead citrate, and examined with an electron microscope. The contralateral, uninjected orbicularis oculi muscles served as controls for these studies.

Results

Normal data

Physiology and lid kinematics

Reflex blinks evoked either by an air puff directed at the orbit or by electrical stimulation of the SO exhibit a similar pattern (Fig. 1A). The blink consisted of a rapid lid lowering (down-phase) followed by a slower lid opening movement (up-phase). A brisk burst of OOemg activity preceded lid closure by 4–7 ms. As described for other species (humans: e.g. Shahani and Young 1973; rats: Evinger et al. 1993), the OOemg response of the guinea pig to SO stimulation exhibited two components: an early R1 response, 5–7 ms after the stimulus, and a late R2 response with a 16–20 ms latency. In humans, the maximum lid velocities achieved during the down-phases and the up-phases were a linear function of blink amplitude (Evinger et al. 1991b). This relationship was also linear for guinea pig blinks. For all guinea pigs, the mean slope of the linear regression line relating maximum velocity and blink amplitude was 44.37°/s per degree (± 3.7) for blink down phase and 23.64°/s per degree (± 3.1) for

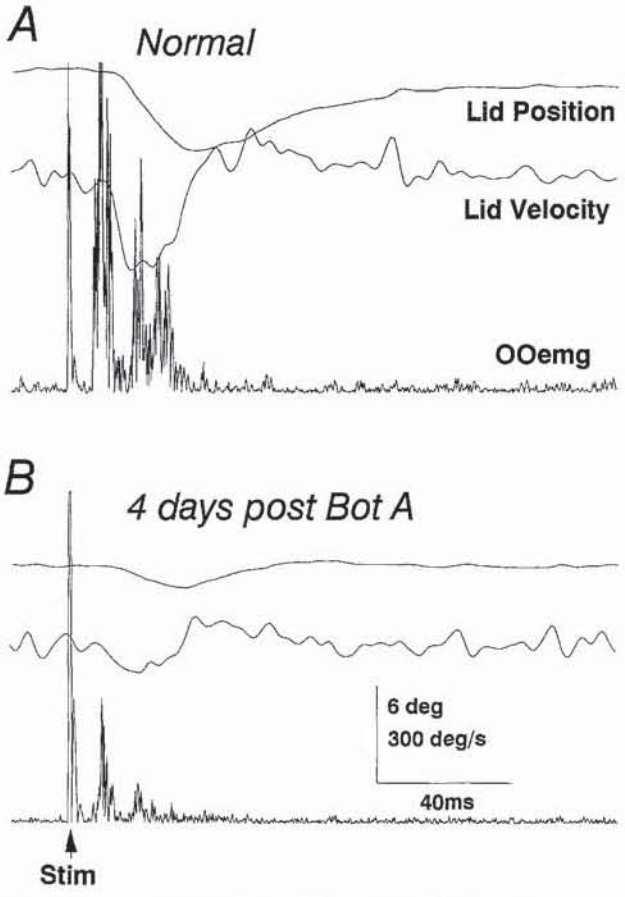

Fig. 1. Upper eyelid position, velocity and rectified orbicularis oculi muscle EMG (OOemg) during a reflex blink after identical electrical stimulation of the supraorbital nerve in a normal, untreated guinea pig (**A**)and 4 days after an injection of Bot A into the upper eyelid (**B**). *Stim*, stimulus artifact

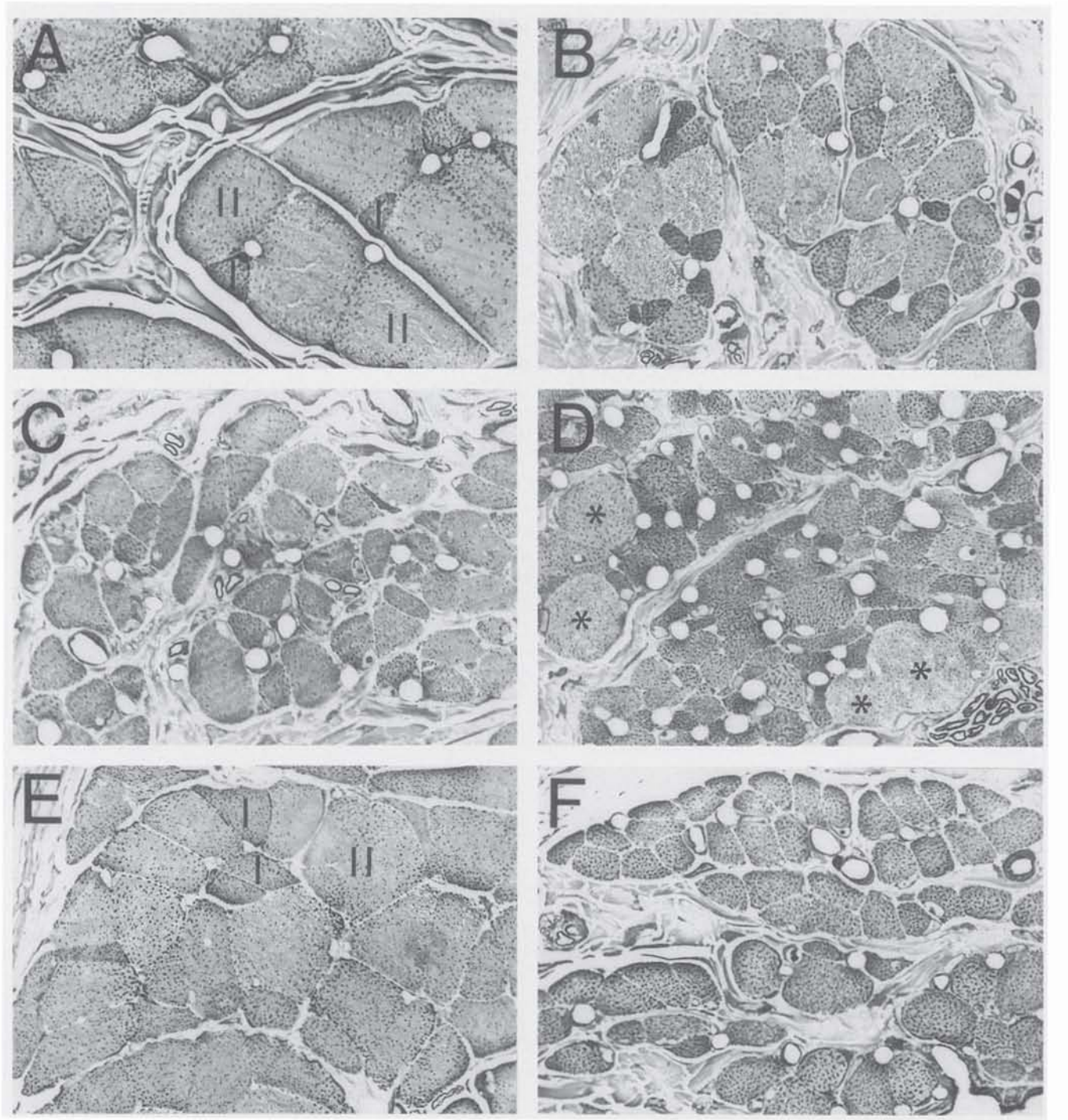

Fig. 2. Light photomicrographs of orbicularis oculi muscles from control (**A**), and botulinum treated muscles after survival times of 4 days (**B**), 12 days (**C**), 21 days (**D**), and 42 days (**E**). Progressive atrophy and recovery of fiber size is illustrated. Type I and II fibers are indicated in **A** and **E**. Type I fibers stain more darkly than type II fibers both before and after Bot A injection. For comparison, muscle 15 days subsequent to motor nerve crush is shown in F. *, Hypertrophied muscle fibers; *I*, type I muscle fiber; *II*, type II muscle fiber. ×515

blink up-phase. Guinea pig blinks achieved higher velocities than human blinks, which had slopes of 29.4°/s per degree and 13.5°/s per degree for down- and up-phases, respectively (Evinger et al. 1991b). Thus, both the blinks and saccadic eye movements of guinea pigs were faster than those of humans (Evinger et al. 1984). As with human blinks, the maximum velocity of the guinea pig down-phase was roughly twice as fast as the maximum up-phase velocity (Fig. 1A). There was only a weak relationship between blink amplitude and blink duration, although the down-phase of the blink typically exhibited a shorter duration than the up-phase. For example, an SO-evoked blink required approximately 105 ms for completion, with the down-phase constituting less than a third of this time (down-phase: 30.5 ms; SD = 6.0; $n = 80$ compared to up-phase: 75 ms; SD = 27.7; $n = 80$).

Orbicularis oculi morphology

The normal guinea pig orbicularis oculi muscle contains the typical complement of skeletal muscle fiber types, including types I, IIA, and IIB. Nevertheless, type II muscle fibers are the dominate muscle type (Fig. 2A). Neuromuscular junctions are randomly distributed throughout the muscle. The fiber type composition of the orbicularis is characteristic of transiently activated muscles, and so is appropriate to the patterned phasic activation of this muscle that occurs during blinks (Fig. 1). Physiological studies (Edström and Lindquist 1973) confirm that the orbicularis oculi is a fast-contracting, but fatigable muscle. At the light microscopic level, the type I fibers are small, with a darker appearance, while type II fibers are larger, and exhibit a lighter staining matrix. By ultrastructural criteria, the type I fibers exhibit abundant mitochondria and large, diffusely defined myofibrils. Type II fibers show variable numbers of mitochondria and small myofibrils that are well defined by the internal membrane system components (i.e., the sarcoplasmic reticulum and t-tubule system).

Effect of Bot A treatment

Physiology and lid kinematics

Bot A treatment significantly reduced reflex blink amplitude and OOemg activity within 1 to 2 days. Within 3 to 4 days after injection there was an almost complete paralysis of the upper lid movement. SO stimulation, identical in intensity to that used before Bot A injection, evoked only a small amount of OOemg activity, primarily in the R1 component. Blink amplitudes achieved only 2–3°, even at stimulus intensities sufficient to evoke blinks well over 10° in amplitude before treatment (Figs. 1B, 3A, B). Since maximum down-phase lid velocity of reflex blinks always fell *below* the regression line for normal animals, Bot A treatment slightly decreased maximum lid velocity at any given amplitude (Fig. 3A). In contrast, there were no significant changes in up-phase maximum velocities following Bot A treatment in five of the six animals (Fig. 3B). Morphological analysis of the levator palpebrae superioris muscle in these six guinea pigs demonstrated that only the animal with abnormally slow up-phase maximum velocities exhibited pathological changes, caused by the spread of Bot A to the levator palpebrae superioris muscle (see below).

The guinea pig results differed from the data for human subjects after Bot A treatment (Manning et al. 1990) in that the slopes of the regression lines for the amplitude-maximum velocity relationship of the guinea pig down-phase blink could not provide a useful measure of Bot A effects. This was because the range of blink amplitudes following Bot A treatment was too small to calculate reliable regression lines for guinea pigs. Nevertheless, since we employed identical blink-evoking stimuli before and after Bot A treatment, and such stimuli elicit constant-amplitude blinks over periods of months (Evinger et al. 1993), it was possible to compare the average blink

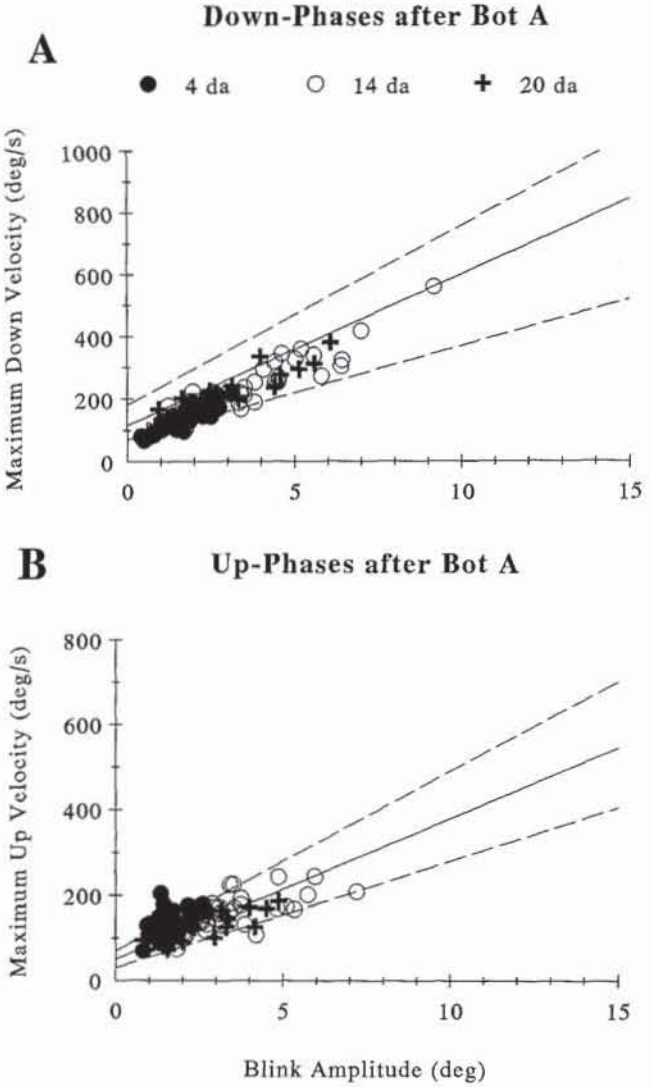

Fig. 3. Maximum lid velocity as a function of lid amplitude for the down-phases (**A**) and up-phases (**B**) at different times following Bot A treatment. The *dashed lines* indicate the range containing 95% of the blinks from the guinea pig before Bot A treatment, and the *solid line* shows the regression line for normal data acquired before Bot A treatment. ● *4 da*, 4 days after treatment; ○ *14 da*, 14 days after treatment; + *20 da*, 20 days after Bot A treatment

amplitude for each day. We calculated the means of the integrated OOemg amplitudes and down-phase amplitudes of all blinks of a daily session and plotted them relative to the average of all pretreatment data. For all guinea pigs, blink and OOemg amplitude declined rapidly within the first 2 days, reaching a minimum (25% of preinjection values) 3 and 4 days after the Bot A injection (Fig. 4). Recovery of OOemg and blink amplitudes began around day 6. Visual inspection of guinea pig lid movements following a blink evoking stimulus revealed clear lid lowering 12 days after Bot A treatment, with a full lid closure appearing approximately 21 days after the injection. Nevertheless, full recovery of blink amplitudes occurred only after 40 days (Fig. 4B). The injection procedure itself was not responsible for this reduction in lid closure, because saline injection into the eyelid did not alter OOemg or blink amplitudes (Fig. 4A).

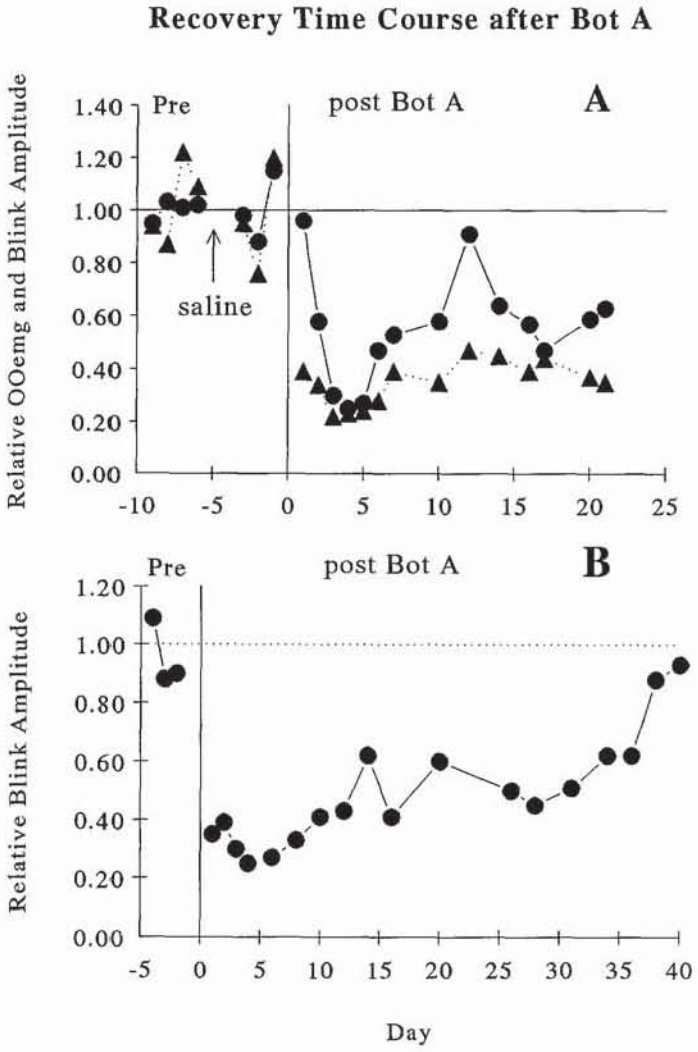

Fig. 4. OOemg magnitude (▲) and down-phase blink amplitude (●) as a function of time before and after a saline injection (**A**, *pre*) and after Bot A treatment for one guinea pig. **B** The mean down-phase blink amplitude (●) as function of time before (*pre*) and after Bot A treatment (*post Bot A*) for two guinea pigs

Orbicularis oculi morphology

Light level changes induced by Bot A. The orbicularis oculi consists of orbital and palpebral components; the palpebral component is partitioned into preseptal and pretarsal divisions. Analyses were restricted to the injected, pretarsal division of the orbicularis oculi. The pretarsal division is thought to participate only in blinks and not in the sustained, voluntary closures of the eyelid (Gordon 1951). Within 4 days after toxin injection, fibers in the vicinity of the injection site were significantly atrophied (Fig. 2B). The toxin effect, however, clearly diminished with distance from the injection site. Because of this apparent diffusion gradient of toxin effect, and the frequent oblique sections produced when sectioning a sphincter muscle, morphometric analyses were not attempted. The micrographs shown in Fig. 2 illustrate the most profoundly altered region that was seen in each case. We utilized the same features for identifying muscle fiber type as we employed for characterizing normal orbicularis oculi muscle. Within the affected muscle region, there was no evidence of a differential response of the three muscle fiber types. By 12 days (Fig. 2C), the degree of muscle atrophy was maximal. Muscle fiber nucleoli were especially prominent in the 4- and 12-day survival cases. By 21 days after toxin injection, many fibers in the vicinity of the injection site remained severely atrophied, but a modest number of fibers now exhibited hypertrophy (Fig. 2D), thereby suggesting the return of muscle function. The 21-day-survival case that had received multiple toxin injections exhibited a wider range of fiber sizes and also presented some degenerating fibers. By 42 days after toxin injection, fiber size had recovered and appeared to be approximately normal (Fig. 2E). In all cases examined, regardless of atrophic changes in muscle or survival time, myelinated axons appeared normal.

Ultrastructural changes induced by Bot A. At the ultrastructural level, fibers in the vicinity of the toxin injection site showed modest to severe myofilament loss from the periphery of individual myofibrils and around the perimeter of individual fibers (Fig. 5A). This effect was most apparent in the 4-day and 12-day survival cases. For the shorter survival times, many of those fibers that showed significant myofilament loss also exhibited structurally normal neuromuscular junctions (Fig. 5A). A smaller number of fibers exhibited rejection of degenerating fiber fragments (Fig. 5B), probably as a result of a longitudinal splitting process. Myofilament loss as a mechanism of reduction of fiber cross sectional area was particularly apparent at 12 days, but was less obvious by 21 days after Bot A injection. By 42 days, though fiber size remained qualitatively smaller than that of the control, the structural integrity of both type I and II fibers was restored as virtually no pathology was noted at the ultrastructural level (Fig. 5C). Muscle regeneration could occur either by the generation of new myofilaments by the original myonuclei or by incorporation of adjacent satellite cells into the fiber. Satellite cell participation in the recovery process was shown by the presence of activated satellite cells or early myoblasts beneath the basal lamina of an existing muscle fiber (Fig. 6D).

In one guinea pig, spread of the toxin altered the active upward movements of the eyelid, which are mediated by the levator palpebrae superioris muscle. Ultrastructural analysis revealed the presence of tubular aggregate-type structures in some fibers of the levator muscle ipsilateral to the toxin injection (Fig. 5D). Rarely, aggregates resulted in the virtual exclusion of myofilaments from individual muscle fibers. Tubular aggregates are formed by overproliferation of sarcoplasmic reticulum and are interpreted as a result of increased intracellular free calcium levels (Gori 1972; Salviati et al. 1985; Spencer and McNeer 1987; Porter et al. 1991).

Toxin-induced alterations in neuromuscular junctions also occurred. Junctions with small preterminal elements that lie either on the fiber surface or in small depressions of the sarcolemma characterized normal Type I fibers (Fig. 6A). After toxin injection, the presynaptic elements at some junctions associated with type I fibers exhibited

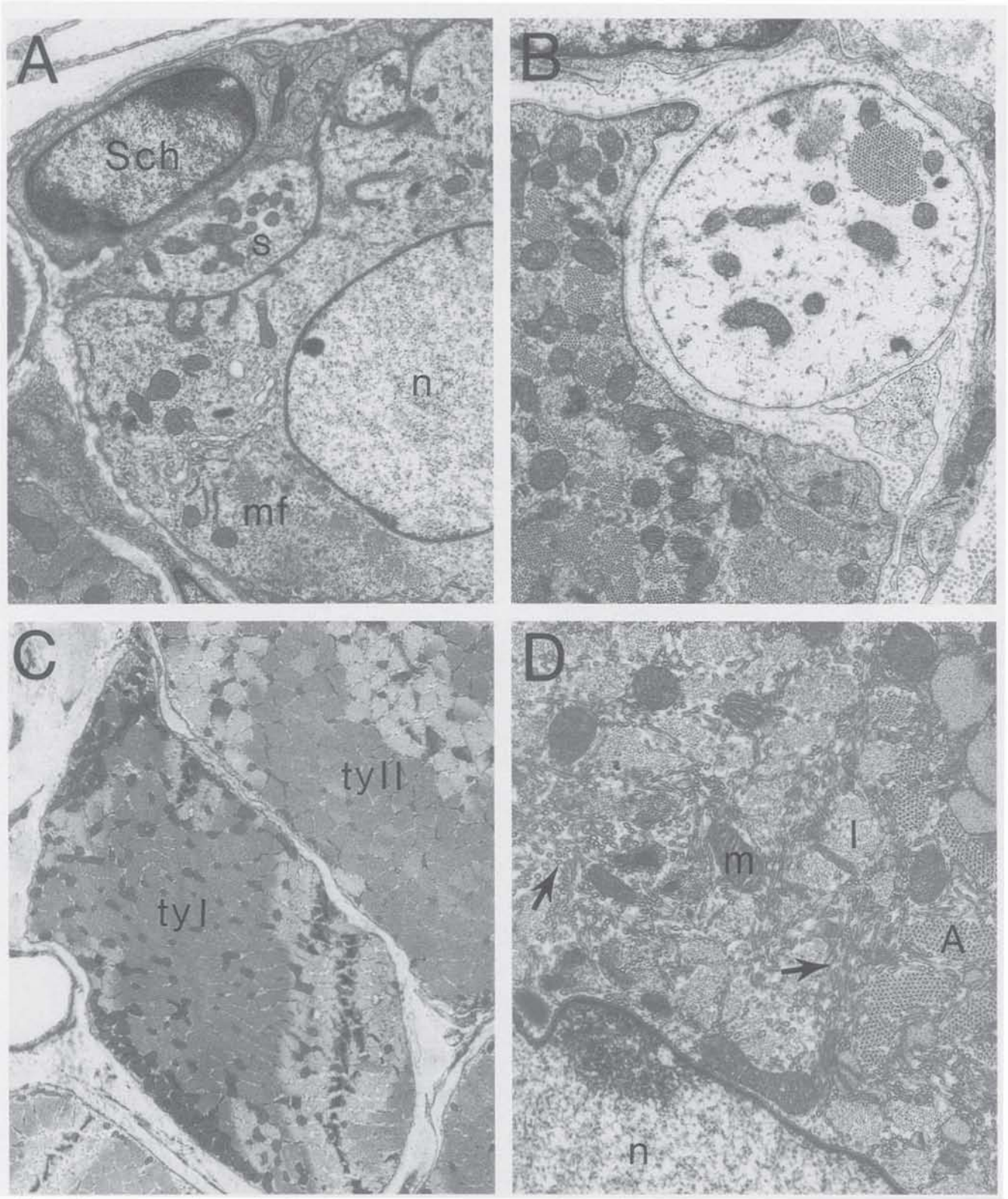

Fig. 5. Electron photomicrographs of botulinum-treated orbicularis oculi (**A–C**) and levator palpebrae superioris (**D**) muscles. An atrophying fiber from a 21-day-survival case shown in **A** has few remaining myofilaments (*mf*), but presents an intact neuromuscular junction (*s*) overlain by a Schwann cell (*Sch*). A myonucleus (*n*) is also indicated. Some fibers exhibit rejection of degenerating fragments, as seen in the 21-day, multiple-injection case (**B**). By 42 days, the integrity of both type I and II fibers is restored, with normal appearance of myofilaments in both A and I bands. **D** A levator muscle fiber from the multiple-injection case in which the toxin spread from the injected eyelid. Note overproliferation of sarcoplasmic reticulum (*arrows*) that resembles tubular aggregates. **A** ×10|500; **B** ×15|000; **C** ×4500; **D** ×22|500

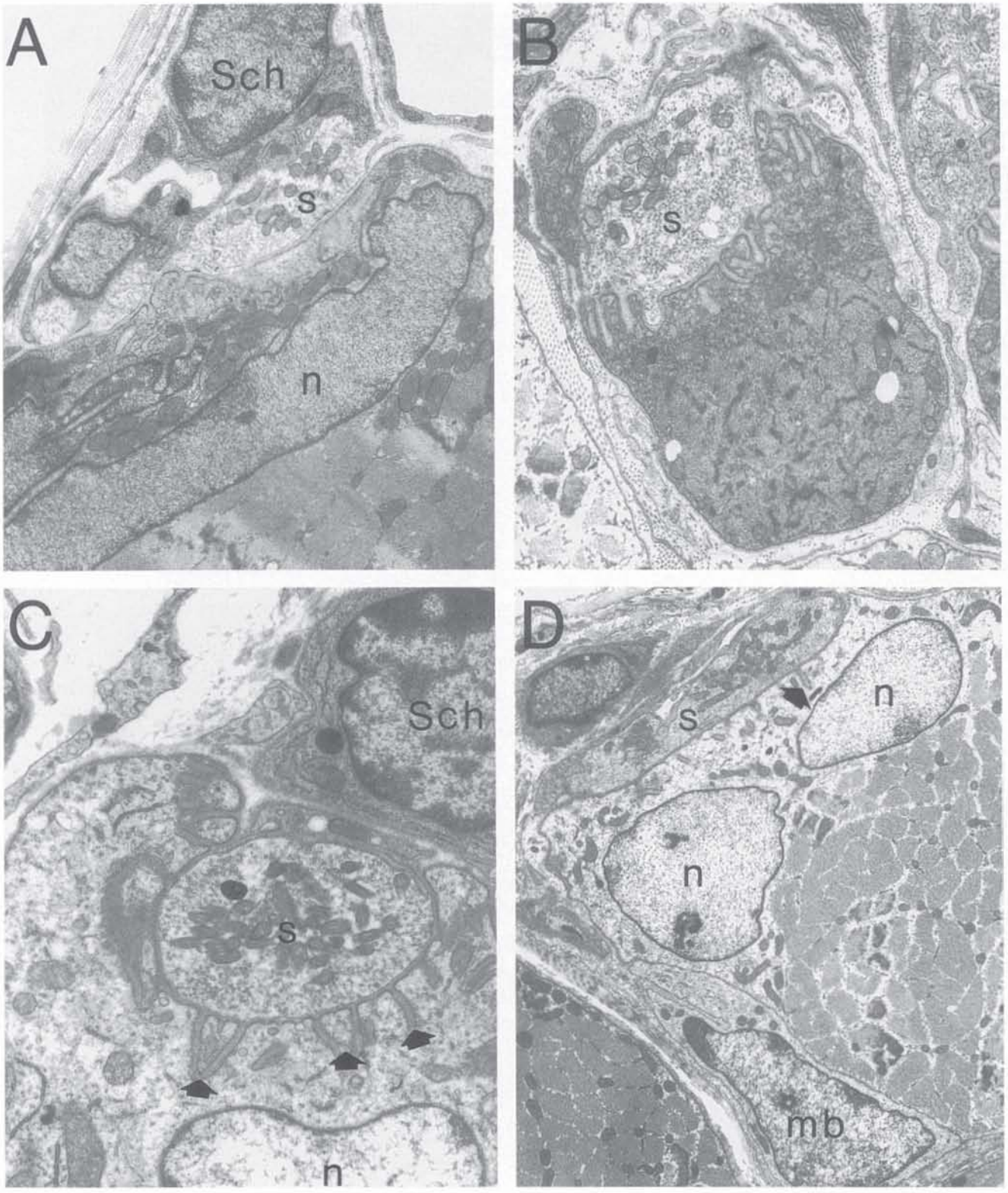

Fig. 6. Electron photomicrographs of neuromuscular junctions (*s*) in normal (**A** and **C**) and botulinum-treated (**B** and **D**) orbicularis muscles. Normal type I fiber junctions (**A**) contrast with the swollen axon terminals associated with a type I fiber 4 days after toxin injection. Likewise, the focal synaptic terminals that are embedded in the surface of type II fibers from control material (**C**) often were replaced by elongated contacts that lie on the fiber surface, as shown in the 42-day survival case (**D**). **A** ×9000; **B** ×10|500; **C** ×13|500; **D** ×6000

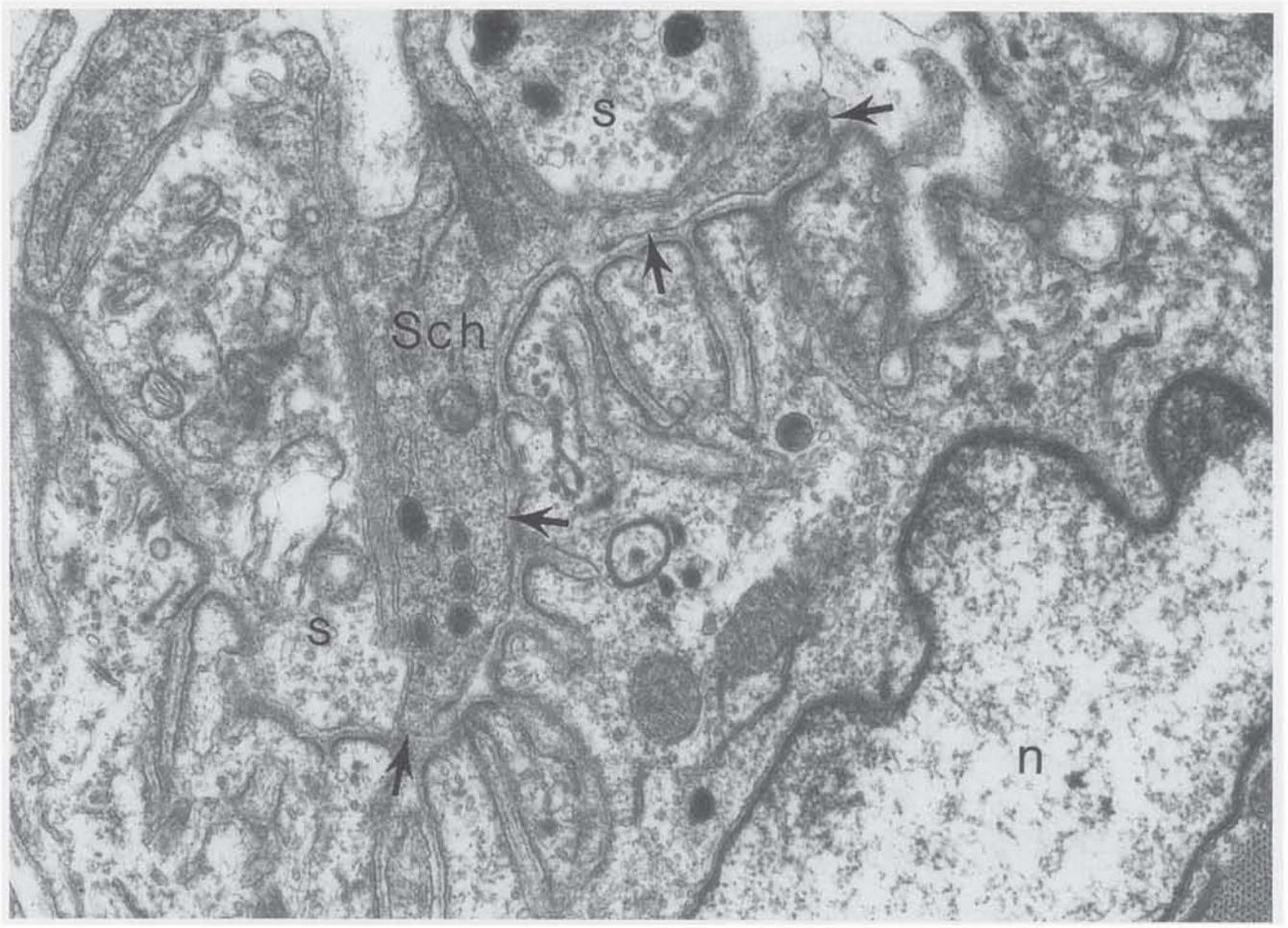

Fig. 7. Electron photomicrograph illustrating evidence of retraction of the axon terminal (*s*) from a neuromuscular junction 21 days after botulinum toxin injection. Note Schwann cell (*Sch*) processes (*arrows*) that intervene either partially or completely between the terminal and surface of the muscle fiber. ×27|000

dramatic swelling (Fig. 6B). Such terminal enlargement is interpreted as evidence of terminal axonal sprouting. Small, rounded presynaptic elements that were deeply embedded in sarcolemmal depressions typified the junctions of normal type II fibers (Fig. 6C). Significant postjunctional folding of the sarcolemma was also apparent in control junctions. By contrast, many of the neuromuscular junctions associated with type II fibers of toxin-treated muscles exhibited elongated presynaptic elements that lay on the fiber surface (Fig. 6D). Often postjunctional folds were few in number at these sites. Occasional neuromuscular junctions exhibited evidence of retraction of the presynaptic elements, with Schwann cell processes intervening between synaptic vesicle-containing axon terminals and muscle-fiber surface (Fig. 7). The hypothesis that terminal and preterminal axon sprouting served to re-establish motor innervation in toxin-treated muscles was reinforced by the finding of axonal growth cones subsequent to Bot A injection, particularly in the 21-day, multiple injection case.

Nerve crush

Physiology and lid kinematics

A crush of the zygomatic branch of the facial nerve caused an immediate, complete paralysis of the upper eyelid. During the first days after nerve crush the animal responded only with movements of its lower lid and retraction of the eyeball to blink-evoking stimuli. As occurred with Bot A treated animals, there was a reduction in maximum blink down-phase velocity (Fig. 8A), and blink and OOemg amplitudes fell to 25% of the normal values in the 1st week following the nerve crush (Fig. 8B). Within the next 10 days OOemg and blink amplitudes rapidly increased, and the nerve-crushed guinea pig regained normal values of blink amplitude and full-lid closure 15 days after the crush.

Orbicularis oculi morphology

Light level changes. At 15 days after facial nerve branch crush, all orbicularis muscle fibers were atrophied (Fig. 2F), pretarsal and preseptal regions of the muscle being

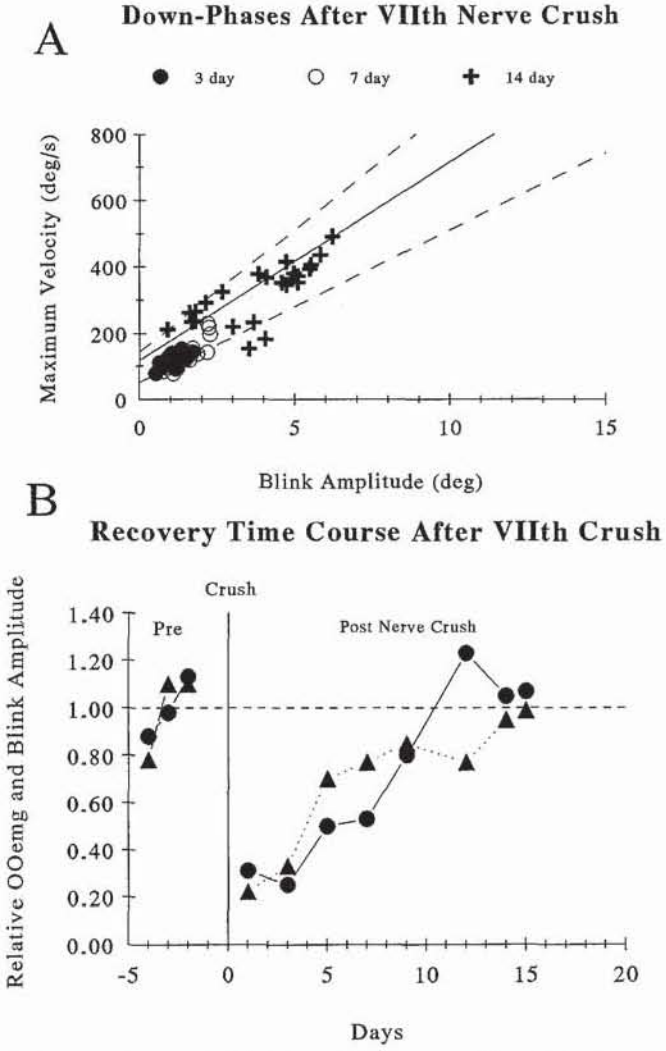

Fig. 8A, B. Changes in blink kinematics after crushing the zygomatic branch of the facial nerve. **A** Maximum lid velocity as a function of down-phase blink amplitude at 3 (●), 7 (○) and 14 (+) days after nerve crush. The *dashed lines* indicate the range containing 95% of the data for this guinea pig before the crush, and the *solid line* shows the best fit linear regression line. **B** Mean OOemg magnitude (▲) and blink amplitude (●) as a function of time before (*Pre*) and after crushing (*Post Nerve Crush*) the zygomatic branch of the facial nerve

equally affected. Muscle morphology most closely resembled that of the 12-day survival Bot A case. Bundles of myelinated axons were interspersed among muscle fascicles, suggesting that functional innervation had been restored. Some former nerve fascicles, identified by the persistence of the enveloping connective tissue sheaths, were occupied by only a few myelinated axons.

Ultrastructural changes. At the ultrastructural level, orbicularis muscle fibers from the nerve-crush case closely resembled those of toxin-treated guinea pigs (Fig. 9). Overall muscle appearance was similar to that seen in the 12-day-survival Bot A case. Muscle fibers were atrophied, often irregular in shape, and exhibited varying degrees of myofilament loss. In addition, elongated areas of nerve-muscle contact, similar to those of toxin-treated muscles (see Fig. 6D), were noted in the nerve-crush case (Fig. 9B). Such contacts contained few synaptic vesicles and large numbers of neurotubules and neurofilaments, and Schwann cell processes often intervened between terminal and muscle surface. These putative newly formed nerve-muscle contacts apparently were preferentially attracted to former synaptic sites, as postjunctional folds were evident in the sarcolemma below such primitive terminals. Postjunctional folds typically appear late during the generation of neuromuscular junctions; the presence of folds in such immature junctions suggests that the folds persisted at a former junctional site. Bundles of presumptive regenerating axons (Fig. 9C) were noted throughout the orbicularis muscle, with both myelinated and small unmyelinated axons interspersed within muscle fascicles. Many of the unmyelinated axons (Fig. 9C) were similar in appearance to those of embryonic muscles (Porter and Baker 1992) in that they often were *not* isolated from one another by Schwann cell processes. The Schwann cells associated with newly formed axons or axon terminals often contained laminated cytoplasmic bodies (Fig. 9B) These are frequently seen in the Schwann cells of axons that have undergone demyelination (Monton et al. 1986).

Discussion

Guinea pigs as a model for Bot A effects on primates

The similarity between primates and guinea pigs in the morphology of the orbicularis oculi and in eyelid kinematics suggests that guinea pigs are an excellent model for interpreting the physiological changes in lid kinematics caused by alterations in muscle morphology after Bot A treatment in humans. The fiber type composition of the guinea pig orbicularis oculi is similar to that previously described in man (Freilinger et al. 1990; Nelson and Blaivas 1991) and monkey (Porter et al. 1989, 1991; McLoon and Wirtschafter 1991). These muscles contain typical skeletal muscle fiber types but predominantly type II fibers in all species. The orbicularis oculi is phasically activated in downward movements of the eyelid during blinks. Blinks exhibit a non-saturating, linear relationship between amplitude and peak velocity in both humans and guinea pigs (e.g., Evinger et al. 1984, 1991b). The predominance of skeletal muscle type II fibers is consistent with this role, since this fiber type exhibits rapid contractions, but is highly fatigable.

Most studies on the effects of Bot A treatment report that the severity and duration are dose-dependent (Scott et al. 1985; however see Dutton and Buckley 1988). Patients typically receive 12.5–50 units/lid per treatment (Frueh et al. 1984; Scott et al. 1985; Tsoy et al. 1985), and in some cases up to 75 units/lid (Dutton and Buckley 1988). On the assumption that 1 unit, the LD50 for mice, is equivalent to 0.4 ng (Geller et al. 1989), and assuming an average human body weight of 70 kg, human patients receive 0.07–0.3 ng/kg per lid treatment. To work from a baseline of visible lid paralysis, we injected guinea pigs with 0.3–0.35 ng/kg per lid, which corresponds to ap-

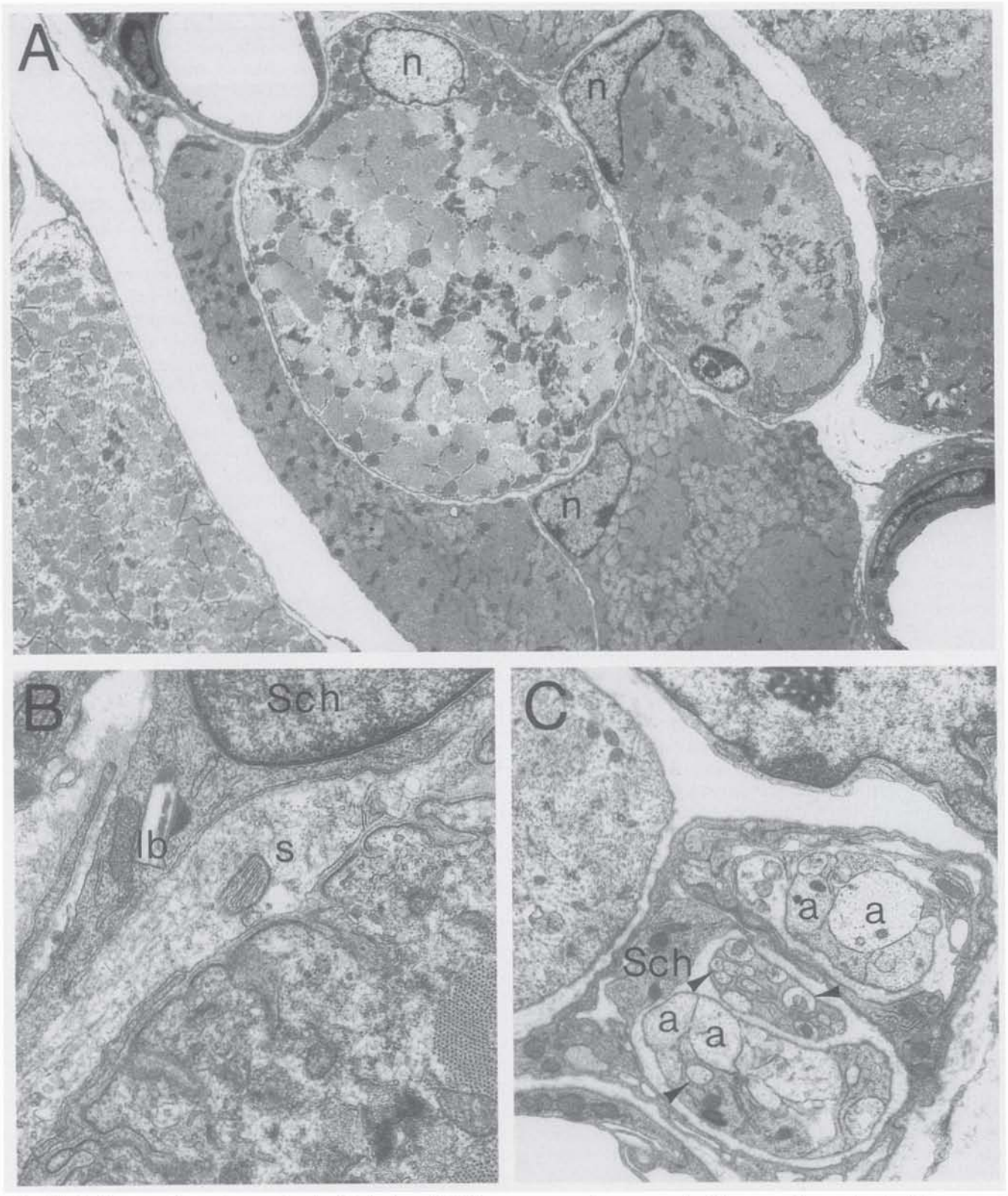

Fig. 9A–C. Electron photomicrographs of orbicularis oculi 15 days after facial nerve crush. Fibers are often irregular in shape and reduced in diameter (**A**). Neuromuscular junctions (*s*) in **B** resemble those of toxin-treated muscles in presenting elongated contacts that lie on the surface of type II fibers. Laminated cytoplasmic bodies (*lb*) were often present in Schwann cells associated with regenerating terminals and axons. Regenerating axons (*a* and *arrowheads*), enclosed in Schwann cell processes (*Sch*), were a prominent feature of the nerve-crush animal (**C**). **A** ×4750; **B** ×22|500; **C** ×10|500

proximately 52–60 units/lid in humans. In human subjects, the relief from spasms afforded by such an injection of Bot A lasts 12–15 weeks (Dutton and Buckley 1988), but this only affected guinea pig lid movements for approximately 6 weeks. Such a difference is consistent with the higher rate of metabolism of rodents relative to primates.

In guinea pigs, Bot A treatment caused a decrease in OOemg activity and blink amplitude, and slowed blink down-phases. These effects were identical to those produced by Bot A in humans (Manning et al. 1990). The reduction of OOemg activity certainly resulted from presynaptic blockade of acetylcholine release at the orbicularis oculi neuromuscular junction by Bot A (Gundersen 1980 for review). Since a linear relationship exists between OOemg magnitude and blink amplitude (Manning and Evinger 1986; Evinger et al. 1991b), blink amplitude must also decrease following Bot A treatment. Despite this correlation in normal animals, Bot A treatment exerted a more profound effect on the OOemg than blink amplitude. Even in the virtual absence of OOemg activity on day 3 and 4 after Bot A treatment, small downward lid movements occurred. These lid movements probably resulted from the passive downward force acting on the upper eyelid (Evinger et al. 1991b; Sibony et al. 1991). When the levator palpebrae superioris relaxes, the passive, downward forces lower the upper eyelid even in patients with a complete seventh nerve palsy (Sibony et al. 1991). Given the similarities between guinea pig and human blinking, the same forces probably acted to lower the upper eyelid of the guinea pig when Bot A produced complete muscle paralysis.

The decrease in the maximum velocity of blink downphase probably occurred from the loss of functional orbicularis oculi muscles and the atrophy of the muscle fibers. Injections of Bot A into the guinea pig eyelid produced significant denervation-like atrophy of all fiber types. In general, these results are similar to those previously observed for the monkey orbicularis oculi (Porter et al. 1991). In both species, all muscle fiber types showed reversible atrophy, and myofilament loss and myofiber fragment rejection produced the reduction in fiber size. Perhaps because of the quantity of toxin injected, monkey orbicularis muscles remained atrophied longer than those of guinea pigs, and also exhibited tubular aggregates in type II fibers, although these were not seen in the guinea pig orbicularis. Tubular aggregates, which are observed in a variety of neuromuscular disorders and were seen in type II fibers only in the monkey orbicularis oculi, are thought to be an adaptive response to elevated free calcium levels (Gori 1972; Salviati et al. 1985). It is not known why these tubular aggregates should be present in toxin-treated monkey orbicularis, but not in that of the guinea pig. The observed myofilament damage, which is consistent with reports of Bot A induction of cathepsin D (Tågerud et al. 1986), a lysosomal enzyme, was as severe as that observed in the facial nerve axotomy case. Muscle damage was most severe in the vicinity of the injection site, with decreasing effects seen with distance from the injection. The location of the lesions and the severity of the toxin's effect upon blinks supported the argument that the palpebral part of the orbicularis oculi was principally responsible for blinking.

It was also possible that an increase in orbicularis oculi stiffness caused by the Bot A treatment, such as has been shown in extraocular muscle (King et al. 1986), contributed to slowing blink down-phases. Alterations in orbicularis oculi passive stiffness, however, should also slow blink up-phases. With the exception of one case, the present data and data from human subjects (Manning et al. 1990) failed to find any decrease in maximum blink up-phase velocity following Bot A treatment. Even though the guinea pig with slow up-phases did not exhibit ptosis, morphological analysis of this case revealed clear Bot A effects on the levator palpebrae superioris (Fig. 5D), similar to that seen in other extraocular muscles (Spencer and McNeer 1987). In human patients, repeated reinjections or higher doses of Bot A occasionally produce a temporary upper lid ptosis caused by spread of Bot A to the levator palpebrae superioris (Scott et al. 1985; Dutton and Buckley 1988). Our single case with contamination of the levator palpebrae superioris demonstrated the strong correlation between lid kinematics and muscle function, and the sensitivity of these measurements for detecting pathological changes in the guinea pig.

Time course

With the combined morphological and physiological approach, we can reconstruct and correlate the modifications in lid movements and OOemg with the morphological alterations in the orbicularis oculi muscle following Bot A treatment. These data can explain the changes in lid motility seen in human subjects. In agreement with the initial relief from lid spasms in human patients (Dutton and Buckley 1988), we found that OOemg magnitude and blink amplitude decreased within the first 2 days after Bot A treatment. The maximal reduction in OOemg magnitude and blink amplitude occurred 3–4 days after Bot A injection, congruous with the significant orbicularis oculi atrophy caused by the loss of contractile myofibrils at this time. The functional recovery of the blink reflex that developed 21 days after Bot A treatment coincided with changes in muscle morphology. Our data, including observations of enlarged synaptic terminals and the presence of growth cones and "primitive" terminals, support the argument that terminal and preterminal sprouting provided for this restoration of muscle function. In addition, the hypertrophy of scattered fibers at 21 days after toxin injection did not support the notion that poisoned neuromuscular junctions recovered, but instead confirmed the hypothesis that sprouting plays an important role in re-establishment of innervation. Other studies provide anatomical and physiological evidence of sprout formation and establishment of functional neuromuscular junctions (Angaut-Petit et al. 1990). In particular, these authors identified newly formed acetylcholine-receptor patches on segments of sarcolemma that were subadjacent to axonal sprouts.

The issue of the relative contribution of sprouting ver-

sus return of function at existing neuromuscular junctions has not been adequately resolved in other studies. Remodeling of mammalian neuromuscular junctions is a normal, continuing process (Robbins and Polak 1988) that is accelerated under experimental conditions. Early studies by Duchen (1970, 1971), noting the formation of sprouts at most neuromuscular junctions in Bot A-treated soleus and gastrocnemius muscles, suggested that synaptic remodeling was important in recovery from Bot A paralysis. Bot A-induced sprouting was described as originating from preterminal axons and synaptic terminals, as opposed to collateral sprouting (for which sprouts arise from nodes of Ranvier). Holds et al. (1990) identified sprouts in botulinum-treated human orbicularis muscles, but indicated uncertainty as to whether they terminated on muscle fibers as functional neuromuscular junctions. Sprouting does appear to be a function of duration of neuromuscular blockade, as multiple injections increase its relative frequency (Alderson et al. 1991). While there was evidence of terminal sprouting in Bot A-treated monkey orbicularis oculi muscles, sprouts were few in number and their contribution to recovery of function uncertain (Porter et al. 1991). Holland and Brown (1981), however, reported a similar correlation between recovery of muscle tension and number of axonal sprouts for the fast peroneus muscle in the mouse.

Maximal blink amplitude reduction was seen at 3 to 4 days after Bot A injection. Significant muscle atrophy was detected at this time, although maximal atrophy was not present until 12 days after toxin injection. While muscle is an elegant structure/function model, and its morphological characteristics depend significantly upon patterned motor activity, the configuration of structural changes observed in the present study consistently lagged behind behavioral observations. Presumably, this observation marks a delay between alterations in innervation and detectable muscle atrophy or recovery. Although blink amplitudes and OOemg activity recovered between days 4 and 21, these parameters remained below normal, in accord with the sustained reduction in fiber size. This time frame of physiological recovery correlated well with the temporal appearance of growth cones and apparently "new" neuromuscular junctions in toxin-treated muscle. Muscle was more adversely affected in the 21-day, multiple-injection case, but not to the extent that a multiple-injection paradigm would be expected to produce more lasting effects upon lid function (cf Porter et al. 1991; Alderson et al. 1991). Indeed, clinical utilization of the toxin does not indicate further improvement after multiple treatments. Complete recovery of blink metrics was not seen until later than 40 days post-injection, thus the correlation between muscle morphology and behavioral recovery was very precise.

In agreement with the hypothesis that recovery from Bot A treatment resulted from axonal sprouting, crushing the facial nerve produced a pattern of physiological and morphological changes similar to that produced by Bot A treatment. In both Bot A treatment and nerve crush, axonal sprouting produced recovery (Figs. 6, 7, 9; Duchen 1971; Brown et al. 1981 for review). Guinea pigs, however, recovered much more quickly from nerve crush (15 days) than from Bot A treatment (40 days), even though the growth rate of axonal sprouts was similar in the two cases (Holland and Brown 1981). Nevertheless, our observations were consistent with the speed of functional recovery from nerve crush obtained in other species and systems. Functional recovery of rat whisker movement occurs 14–20 days after a facial nerve crush (Vaughn 1990). Similarly, full tension of the soleus muscle returns 13 days after a sciatic nerve crush (Tonge 1974). Therefore, the present physiological and morphological findings support the idea that the more rapid recovery after nerve crush was due to reinnervation of original endplates by crushed axons compared to the formation of new functional endplates at extrajunctional sites in Bot A-treated muscles (Holland and Brown 1981; Rich and Lichtman 1989).

Our investigations demonstrate that Bot A treatment and nerve crush generate a transient reduction of OOemg magnitude and blink amplitude as well as a slowing lid closure. Since transient changes of lid motility correlate well with morphological changes of the orbicularis oculi muscle, the guinea pig blink reflex is an excellent system for testing procedures that prolong the beneficial effects of Bot A in treating lid spasms (Brown et al. 1977; Tsujimoto and Kuno 1988; Booth et al. 1990). While the origin of Meige's disease, blepharospasm and hemifacial spasms are poorly established, all these diseases manifest themselves as hyperexcitability of blink circuits, resulting in spontaneous clonic and tonic lid contractions (e.g. Berardelli et al. 1985; Valls-Sole and Tolosa 1989). Bot A increases the threshold for the development of lid spasms by weakening the overactive orbicularis oculi. Nevertheless, since weakening the orbicularis oculi with facial nerve damage or Bot A treatment *increases* the excitability of blink circuits (Evinger et al. 1991a), it is critical to investigate further the full range of Bot A effects on lid spasms.

In summary, these studies indicate that Bot A produces a denervation-type atrophy in the orbicularis oculi muscle. All muscle fiber types ultimately recover, with no long-standing consequences of the transient denervation and recovery is the result of preterminal and terminal axonal sprouting that re-establishes functional innervation. These findings are consistent with clinical observations that Bot A produces only temporary relief in patients with essential blepharospasm. It is likely that the efficacy of Bot A in treatment of blepharospasm could be improved through co-application of agents that suppress terminal sprouting.

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