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Axotomy-like Changes in Cat Motoneuron Electrical Properties Elicited by Botulinum Toxin Depend on the Complete Elimination of Neuromuscular Transmission

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The electrical properties of cat medial gastrocnemius (MG) spinal motoneurons were studied 14–21 d following injection of type A botulinum toxin (BTX) into the MG muscle. Treated MG muscles were atrophic, displayed pronounced fibrillation activity, and were markedly but not completely paralyzed. MG motoneuron electrical properties from animals with the highest MG muscle-twitch forces (>20 gm) appeared normal, while motoneuron properties from animals with the lowest MG muscle-twitch forces (<10 gm) exhibited axotomy-like changes, though these changes were less pronounced than after axotomy itself. No changes in the axonal conduction velocity were observed, however. Motoneuron connectivity with MG muscle fibers was determined following intracellular stimulation of MG motoneurons by averaging EMG signals from 3 or 4 pairs of recording electrodes inserted into the BTX-treated MG muscles. Normal electrical properties were observed among motoneurons in which detectable EMG activity linked to the intracellular stimulation pulse was observed. The level of this connectivity, however, indicated that a relatively small number of muscle fibers were activated by individual motoneuron action potentials. Axotomy-like changes of electrical properties were observed in MG motoneurons that could not be associated with detectable EMG activity in the BTX-treated MG muscle following repeated trials of intracellular stimulation. These results indicate that the existence of effective neuromuscular transmission at a small number of motor terminals is sufficient to prevent the appearance of axotomy-like changes in motoneuron electrical properties, and that the absence of such transmission at all motor terminals is associated with the appearance of axotomy-like changes. The results suggest that the effects of axotomy itself on motoneuron properties may be based upon the loss or elimination of a potent interaction between muscle and motoneurons normally mediated by neuromuscular transmission.

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Within adult motor nuclei, there exists a range of motoneuron electrical properties. Across this range, these properties are closely matched to the mechanical properties of the muscle fibers that motoneurons innervate (Burke, 1981; Fleshman et al., 198·), and this matching likely plays an important role in the orderly operation of motoneuron pools and efficient force generation by whole muscles (Henneman and Mendell, 1981; Gustafsson and Pinter, 1985). A question of interest is how this functional organization within motoneuron pools is maintained. One clue is provided by the response of the motoneuron pool to axotomy, in which marked changes in the distributions of properties occur (Kuno et al., 1974a; Gustafsson, 1979; Gustafsson and Pinter, 1984b; Foehring et al., 1986; Pinter and Vanden Noven, 1989). Recovery of normal properties and the pattern of relationships among properties occurs when reinnervation is allowed (Kuno et al., 1974b; Gordon and Stein, 1982; Foehring et al., 1986) and does not occur when reinnervation either fails (Gordon and Stein, 1982; Foehring et al., 1986) or is prevented (Goldring et al., 1980; Pinter and Vanden Noven, 1989). These findings suggest that processes associated with normal muscle innervation are involved in regulating and/or specifying the normal distribution pattern of motoneuron properties, and that the effects of axotomy on motoneuron properties are related to the loss of muscle innervation.

Other evidence supports the notion that muscle activity or the metabolic state of muscle is involved in the regulation of motoneuron properties (Gallego et al., 1979). Kuno and coworkers found that chronic tetrodotoxin (TTX) application to the cat soleus muscle nerve produced axotomy-like changes in the afterhyperpolarization (AHP) duration of soleus motoneuron electrical properties, and that electrical stimulation of the muscle nerve distal to the TTX application site prevented this change (Czeh et al., 1978; see also Munson et al., 1985). The results of experiments employing botulinum toxin (BTX), which blocks release of ACh (Simpson, 1989), imply a role for neuromuscular transmission in the regulation of motoneuron properties. Watson (1969) found that blockade of neuromuscular transmission by BTX provokes metabolic changes in rat facial motoneurons that are observed following axotomy; however, Watson emphasized the regenerative response of motor terminal sprouting after BTX, rather than the block of ACh release per se, as the primary event leading to axotomylike changes after BTX. More recently, Hoffman et al. (1988) have found that intramuscular injection of BTX also produces a decline in neurofilament protein expression in motoneurons and an associated onset of somatofugal axonal atrophy. Both of these effects are also observed after axotomy.

In the present study, we examined the effects of intramuscular injections of BTX on the electrical properties of cat motoneurons to determine whether these properties are also affected after blockade of ACh release. The electrophysiological approach used in this study offered the important advantage of relating the properties of individual motoneurons to the physiological status of the neuromuscular synapse.

This work has appeared in abstract form (Pinter et al., 1989).

Materials and Methods

Initial procedures. Young adult cats of either sex and weighing 2.5–3.2 kg were used in this study. Experimental animals were first anesthetized with 1.0–2.0 ml of a mixture of ketamine (99 mg/ml) and acepromazine (1 mg/ml). Under sterile conditions, the left medial gastrocnemius (MG) muscle was exposed in 10 animals and injected at multiple sites with a solution of type A BTX (Oculinum, Smith-Kettlewell Eye Institute, San Francisco, CA) in 1.0–1.5 ml sterile saline. Each nanogram of BTX in this form equals approximately 4 mouse LD_{50} units. In most animals (7), approximately 10–15 ng (40–60 mouse LD_{50} units) of BTX was used. Two animals received injections of 5 ng BTX (20 LD_{50} units), and 1 received 25 ng of BTX (100 LD_{50} units). Using the wet weight of the untreated, contralateral MG muscle determined on the day of the acute experiment as an estimate of the original weight of the BTX-treated MG muscles, these doses correspond to about 2.0–13.0 mouse LD_{50} units per gram of MG muscle tissue. These BTX doses are considerably lower than amounts used previously in cats (Thesleff, 1960), rats (Bambrick and Gordon, 1987), mice (Holland and Brown, 1981), and chick embryos (Pittman and Oppemheim, 1978) and were used to limit the possibility of systemic botulism. Two animals were injected with 1.0–1.5 ml saline only, as controls for the injection procedure. Prior to closure of the wounds, the exposed tissues were irrigated with sterile saline to clear excess BTX solution. Penicillin was administered for the first 3 postoperative d to prevent infection. In all cases, recovery from anesthesia was uneventful, and in no cases were signs of systemic botulism observed. All treated animals behaved and moved normally.

Procedures for acute experiments. Following a variable postinjection interval, animals were anesthetized with 35–40 mg/kg pentobarbitone. Supplemental doses of anesthetic were administered during the course of the acute experiment through a venous catheter in order to maintain pupillary constriction and areflexia following paw pinch. Blood pressure and end-tidal CO_2 were monitored with arterial and tracheal cannulae, respectively. Occasionally, blood pressure was maintained above 80 mm Hg by a slow infusion (approximately 5 ml/hr) of a half-normal Ringer's solution with 5% dextrose. All animals respired naturally.

The left MG muscle was exposed, and a steel hook was attached to the severed tendon to enable connection of the muscle to a strain gauge for force recording. The left MG nerve was located, dissected free of connective tissue, and mounted on a pair of bipolar stimulating electrodes. The left lateral gastrocnemius-soleus (LGS) muscle nerve was severed and mounted on a bipolar stimulating electrode. In the first experiment of this series, 1 pair of braided stainless-steel wires was inserted into the MG muscle for recording of electromyographic (EMG) activity. In the remainder, 3 or 4 pairs of EMG electrodes were inserted into the MG muscle. No EMG electrodes were used in saline control experiments.

The spinal cord was exposed by laminectomy of the L_4–L_6 vertebral segments. All dorsal and ventral roots remained intact. All exposed tissues were covered with mineral oil. Rectal temperature was regulated at 37–38°C by a heating pad and infrared lamps connected to an electronic control circuit.

Recording procedures. Following placement of the animal in a frame that immobilized the lumbar vertebral column, the MG whole-muscle twitch tension was measured isometrically (100 gm preload) after electrical stimulation of the MG nerve on the BTX-treated side.

Intracellular records were obtained from antidromically identified MG motoneurons using glass micropipettes filled with 3.0 M KCl. All intracellular records were digitized on line and stored for subsequent analysis. Data were obtained only from motoneurons with antidromic action potentials exceeding 80 mV. Rheobase current was determined as the minimal amount of current (50-msec pulse) needed to evoke an action potential (Gustafsson and Pinter, 1984c). The afterhyperpolarization (AHP) duration was measured as the time interval between the point where the voltage trajectory first crosses the baseline following an intracellularly evoked action potential and the return of the postspike trajectory to the baseline. The longest "membrane" time constant was obtained from the voltage decay following 0.5-msec, 20-nA hyperpolarizing current pulses (Gustafsson and Pinter, 1984a). Axonal conduction velocities were also measured. Input resistance was determined using 1–3-nA hyperpolarizing current pulses (50-msec duration). A measure of the over-, undershoot ("sag") membrane property (Ito and Oshima, 1965) was also obtained from these pulses by taking the ratio of the maximum and steady-state membrane voltages (Gustafsson and Pinter, 1984a; Pinter and Vanden Noven, 1989). A sag ratio close to 1.0 indicates the absence of measurable sag in the voltage response.

During initial studies of animals 14–21 d after BTX injections, we attempted to record motor unit mechanical data after the collection of electrical property data using intracellular stimulation of MG motoneurons with suprathreshold 0.5-msec depolarizing pulses, but we were not able to detect motor unit force. Thus, attempts to record motor unit force in later experiments using these postdose intervals were not systematically performed. In saline control experiments and in the 1 animal studied 140 d after BTX, motor unit force recording was attempted for each impaled MG motoneuron.

For the detection of unblocked neuromuscular synapses capable of activating muscle fibers in animals studied 14–21 d after BTX injections, we adopted the alternative approach of obtaining averages from each pair of EMG recording electrodes placed in the MG muscle, synchronized with the intracellular stimulating pulses. Because of the large amount of background noise generated by muscle fiber fibrillations in BTX-treated muscles (Josefsson and Thesleff, 1961), an average of at least 50–100 trials per electrode pair was needed to reveal an EMG signal synchronized to the motoneuron stimulus pulse. In some cases that failed to reveal a clear EMG signal after initial attempts, averages of as many as 300 trials per electrode pair were repeated. Intracellular recording stability places limits on the number of such trials that can be performed. The uncertainty associated with negative findings with EMG averaging are discussed below.

At the conclusion of each experiment, the wet weights of the BTX-treated MG muscle and the contralateral untreated MG muscle were determined. Wet weight of the lateral gastrocnemius (LG) muscles on both sides were also determined.

Results

The results are based on data obtained from 8 BTX-treated animals and 2 saline-injected animals. Intracellular records from 144 MG motoneurons (mean antidromic spike, about 94 mV) were obtained from 2 BTX-treated animals studied 14 d after BTX injection and 5 animals studied 21 d after BTX. An additional 55 MG motoneurons from these experiments yielded only axonal conduction velocity data. One animal was studied 140 d after BTX (19 motoneurons). Two animals were studied 14 d after saline injections (18 motoneurons total). Insufficient intracellular sampling was obtained in 2 animals studies 21 and 42 d after BTX treatment.

Descriptions of whole MG muscle. On the day of the intracellular experiment, visual inspection and EMG recordings revealed that each of the BTX-injected MG muscles studied 14–21 d after the injection was atrophic and exhibited pronounced fibrillations over the entire muscle surface (Josefsson and Thesleff, 1961). On average, the injected muscle wet weight was about 70% of the uninjected MG muscle on the right side 14–21 d after injection. Atrophy (about 60%) was also present in the MG muscle studied 140 d after BTX. In contrast, the wet weights of the LG muscles on the treated side in all these experiments were identical to the uninjected side, the mean weight ratio (treated/untreated) being 0.98. This indicates that the effects of BTX were confined to the MG muscle. A portion of each of the effects described above may be related to the injection procedure itself because both saline-injected MG muscles displayed small amounts of fibrillations, and in 1 case, the wet weight of the

injected muscle was about 80% of the uninjected, contralateral muscle.

The whole-muscle twitch forces of BTX-injected muscles were clearly depressed, but complete paralysis was not observed. The normal MG muscle in cats weighing 2.5–3.2 kg can be expected to produce 150–200 gm for each gram of muscle weight (Spector et al., 1980). Using 175 gm force per gram MG wet weight and the weight of the contralateral, uninjected MG muscle in each experiment as a guide, an estimate of the expected force range would be 800–1600 gm, with a mean of about 1250 gm force. For animals investigated 14–21 d after BTX injections, the observed whole-muscle twitch forces ranged between 3 and 86 gm, with a mean of about 21 gm (N = 7; median, 5 gm). This indicates that the percent denervation probably lies in the range of 93.0–99.0%, with an estimated mean of about 98%. The extensiveness of the neuromuscular blockade present in these experiments is also illustrated by the fact that the largest whole-muscle twitch force encountered (86 gm) is comparable to the largest *single motor unit* twitch forces observed in the cat gastrocnemius muscle (Burke et al., 1973). The 1 MG muscle studied at 140 d post-BTX had a twitch tension considerably greater (666 gm; 57% estimated denervation), presumably because of reinnervation. By contrast, saline-injected MG muscles generated 1.4 and 1.3 kg of twitch force (160 and 168 gm force per gram of MG tissue, respectively). This indicates that the paralysis is attributable to BTX and not to the injection procedures.

In the group of 7 animals studied 14–21 d after BTX, there was no association between either the extent of atrophy or the estimated percent denervation and the dose of BTX. For example, 1 MG muscle injected with 10 ng BTX had a twitch force of 25 gm 21 d later (98% denervated), while another showed 86 gm force after 15 ng BTX (93% denervated). The wet weights of the uninjected, contralateral MG muscles in these cases were similar (7.6 and 7.2 gm, respectively), suggesting that preinjection muscle sizes were also comparable. In another case, we found it possible to achieve a virtually complete paralysis (approximately 3 gm final tension) of a 6.6-gm MG muscle (estimated weight from contralateral muscle) with as little as 5 ng BTX or just over 1 mouse LD_{50} (99.6% denervated). It is our impression that, at the dose levels used in the present study, a thorough infiltration of the muscle with the BTX solution is the most important factor determining the extent of the final paralysis after BTX injection. Our results also indicate, however, that for practical purposes, it does not seem possible to achieve a complete paralysis of the cat MG muscle by these methods. The average weight of all uninjected MG muscles in this study was 7.3 gm, and available evidence indicates that the cat MG muscle is innervated by 300–400 motoneurons and that each of these contacts 500–700 muscle fibers (Burke, 1981). With the large number of motor terminals thus implied, the large size of the muscle, and the relatively small BTX doses, it seems quite plausible that some synapses remained unaffected by BTX.

Motoneuron properties. Mean rheobase currents from experiments with the lowest whole-muscle twitch force (<10 gm) tended to fall below the range of normal mean rheobase, while experiments with higher twitch forces (>20 gm) displayed mean rheobase currents within the normal range, as did both saline control experiments. This may be seen in Figure 1, which shows a plot of the mean rheobase current obtained from at least 10 MG motoneurons for post-BTX dose intervals of 14–21 d. The horizontal lines represent the limits of mean rheobase values of normal MG motoneuron pools obtained from another study

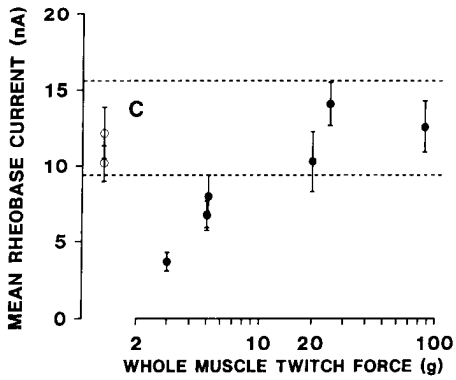

Figure 1. Plot of mean rheobase current versus twitch force of BTX-treated MG muscles. Data from BTX-treated muscles are indicated by *solid symbols.* Note that 3 data points are present at 5 gm force. Data from 2 saline-injected control muscles are indicated by *open symbols* (labeled *C*). Error bars indicate ±1 SEM. *Dotted lines* represent absolute range of mean rheobase current for normal MG motoneurons from Pinter and Vanden Noven (1989).

(Pinter and Vanden Noven, 1989), and the open symbols represent mean rheobase current from saline controls (labeled "C"). The distributions of rheobase current from individual BTX experiments also tended to segregate according to the twitch force. This may be seen in Figure 2*A*, which shows the cumulative distributions from experiments with twitch force <10 gm by open symbols and >20 gm by solid symbols. Experiments with twitch force <10 gm include 2 each from 14 and 21 d, while all experiments (3) with twitch force >20 gm occurred 21 d after BTX injection. Statistical analysis (Kruskal–Wallis analysis of variance) failed to reveal significant differences among the distributions within these 2 groups ($p > 0.10$), so data were pooled. The distributions of pooled data are shown in Figure 2*B*, where the median values are 5.1 nA (<10 gm) and 12.6 nA (>20 gm). The difference between these distributions was significant ($p < 0.01$; Mann–Whitney U test). It is unlikely that the injection procedure itself plays any role in these differences because the median rheobase currents from saline controls were 11.0 and 13.3 nA

The qualitative difference between the rheobase distributions shown in Figure 2*B* is similar to the difference observed between normal MG motoneurons and MG motoneurons axotomized 21 d earlier by peripheral MG nerve transection. For comparison, the median values for axotomized and normal motoneurons from the study of Pinter and Vanden Noven (1989) were 3.1 and 11 nA, respectively. It should be noted, however, that while the distribution of data from animals with greater MG twitch force (>20 gm) is nearly identical to that seen in normal motoneurons, the distribution of data from the lower-force group (<10 gm) contains rheobase currents that are considerably greater than those observed 21 d after axotomy. Thus, the difference between the rheobase distributions of Figure 2*B* is less pronounced than the difference between axotomized and normal motoneurons.

Axotomy-like differences were also observed in the distributions of other motoneuron electrical properties when data were grouped according to whole muscle twitch force, though these differences were also less pronounced than in comparisons of normal and axotomized MG motoneurons. The clearest difference was observed in the distributions of the sag ratio shown in Figure 3*A* ($p = 0.02$). The distributions of input resistance

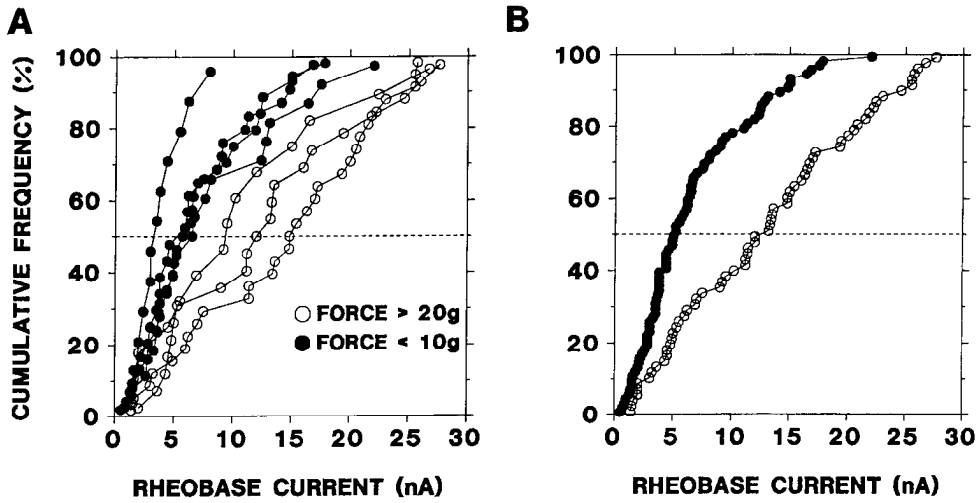

Figure 2. *A*, Cumulative histograms of rheobase current of MG motoneurons studied 14–21 d after BTX injections. Data from experiments in which MG whole-muscle force <10 gm and >20 gm are indicated by *solid* and *open symbols*, respectively. Median values can be read at the intersection of the *horizontal dotted line* (50th percentile) with each histogram. *B*, Histograms of pooled data from *A*. In cumulative histograms, *dotted horizontal line* marks position of 50th percentile.

(Fig. 3*B*) and AHP duration (Fig. 3*C*) also showed significant differences ($p = 0.01$), while the membrane time constant distributions (Fig. 3*D*) showed a small axotomy-like difference that was not statistically significant ($p > 0.05$). An exception to this axotomy-like pattern was found in the distributions of axonal conduction velocity, in which no difference between the data groups with twitch force <10 and >20 gm was observed (Fig. 4).

Motoneuron connectivity. The lower twitch forces in the <10 gm data group suggest that the muscles in this group had been more extensively "denervated" by the BTX injections. However, the estimate of mean denervation in this group (over 99%) was not significantly greater than the estimate for muscles with twitch force >20 gm (about 97%). Nevertheless, MG motoneuron connectivity data obtained using EMG signal averaging indicate that the MG motor nuclei supplying muscles generating >20 gm force contained significantly more individual motoneurons with unblocked motor terminals than nuclei supplying muscles generating <10 gm force. Sampling from implanted EMG electrode pairs was obtained from 96 of the 144 impaled MG motoneurons studied 14–21 d after BTX injections and in all motoneurons studied in 1 animal 140 d after the BTX injection. Data from the first BTX experiment, in which only 1 pair of EMG electrodes was used, are not included in this analysis. Within the 14–21-d data, 44 MG motoneurons (46%) were obtained from experiments with twitch force >20 gm, while 52 motoneurons (54%) were obtained from experiments with twitch force <10 gm. Evidence of unblocked neuromuscular synapses was obtained in a total of 35 MG motoneurons. Examples of averaged records from a motoneuron in which the EMG signal appeared in only 1 of 4 implanted electrode pairs are shown in Figure 5*A*. In this case, the MG muscle had been injected with BTX 21 d earlier and displayed a twitch tension of 86 gm, the largest in the 14–21-d postBTX group of animals. The EMG signal may be seen in trace 4 (shown enlarged in Fig. 5*C*). Although we did not test for the presence of a motor-unit twitch response in this example, it was undoubtedly quite small. This point is illustrated by the example records shown in Figure 5, *B* and *D*. These were obtained from a motor unit studied 140 d after BTX (MG whole-muscle twitch force, 666 gm). The averaged EMG records (Fig. 5*B*; same gain as in *A*) illustrate the presence of EMG activity in 3 of 4 electrodes, but the twitch force of this unit was only about 1 gm (Fig. 5*D*). In 61 motoneurons studied 14–21 d after BTX, no EMG signals were detected after complete sampling from all implanted EMG electrodes. During all EMG averaging runs, the effectiveness of the intracellular stimulation of the motoneuron cell body was visually verified on the oscilloscope screen, and in each case, a full IS-SD spike was observed, indicating activation of both the motor soma and the motor axon initial segment. Nevertheless, it might be argued that motor axonal action potentials did not invade motor terminals in the cases where EMG signals were not detected. This possibility is unlikely based upon previous evidence that BTX (type D) does not compromise action potential invasion of motor terminals (Harris and Miledi, 1971).

Table 1, column 1, shows that the frequency of occurrence of MG motoneurons with unblocked neuromuscular synapses in

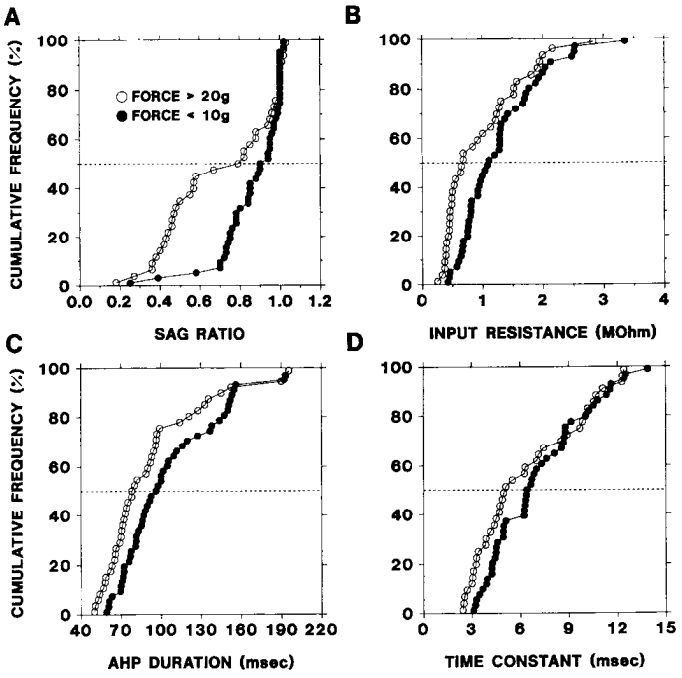

Figure 3. Cumulative histograms of MG motoneuron electrical properties grouped according to twitch force of BTX-treated MG muscle. *A*, Sag ratio. *B*, Input resistance. *C*, AHP duration. *D*, Time constant.

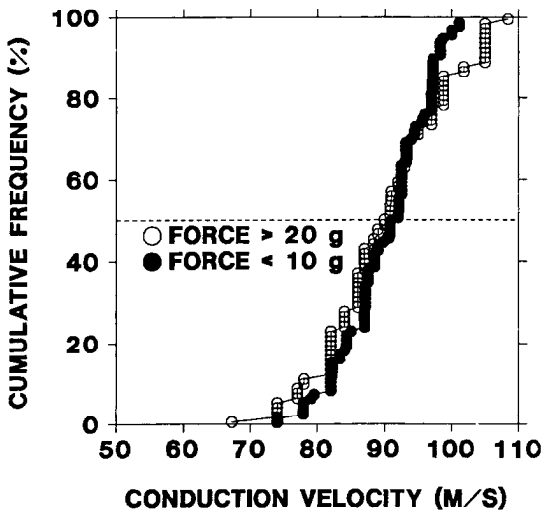

Figure 4. Cumulative histograms of MG axonal conduction velocity grouped according to twitch force of BTX-treated MG muscle.

experiments with twitch force >20 gm was more than twice as great as in experiments with twitch force <10 gm, while column 2 shows that the distribution of frequencies was essentially reversed when EMG signals in the MG muscle could not be detected. In contrast, EMG signals were detected for all 19 motoneurons studied 140 d after BTX.

We suspect that the number of motoneurons possessing unblocked neuromuscular synapses capable of activating muscle fibers 14–21 d after BTX has been underestimated. In 20 of the 35 cases in which EMG signals were detected, complete sampling was obtained from all implanted EMG electrode pairs. In 16 of these 20 cases, the EMG signal appeared in only 1 of 3 or 4 of the electrode pairs (Fig. 5*A*), while in 3 cases, the signal appeared in 2 adjacent electrodes, with one signal being considerably larger than the other. For comparison, in the 140-d animal, 15 out of 19 motoneurons evoked signals in all 4 implanted electrodes, while 3 out of 19 evoked signals in 3 of 4 electrodes (Fig. 5*B*), and 1 out of 19 provided signals in 2 of 4 electrodes. The apparent localization of unblocked synapses in the 14–21-d experiments raises the possibility that such connections may escape detection if they are too small or are located too distantly from an electrode pair. Therefore, the number of motoneurons with unblocked synapses may have been higher if more electrode pairs had been used in each experiment. It thus seems likely that the collection of 61 MG motoneurons in which EMG signals were not detected contains an unknown proportion of neurons possessing some unblocked synapses. However, the trend for the EMG signals to appear in relatively restricted regions of the muscle, and the low whole-muscle twitch forces observed 14–21 d after BTX administration, indicate that the vast majority of neuromuscular synapses were probably blocked even in those motoneurons in which evidence of unblocked synapses could be obtained (Fig. 5*A*).

Axotomy-like differences in the distributions of motoneuron electrical properties were apparent when data were grouped according to the presence or absence of detectable synaptic connections with muscle fibers. In Figure 6*A*, it may be seen that the cumulative distribution of rheobase current of motoneurons with no detectable synaptic connections (solid symbols) is shifted to the left of the distribution of rheobase of motoneurons

Table 1. Frequency of occurrence of MG motoneurons associated with (connected) and not associated with (unconnected) detectable EMG activity in BTX-treated MG muscles following intracellular stimulation

	Connected	Unconnected	*N*
>20 gm	71.4%	31.0%	44
<10 gm	28.6%	69.0%	52
N	35	61	96

Data are grouped according to the twitch forces of BTX-treated MG muscles 14–21 d after BTX injections. Note that frequencies total 100% only when summed by columns.

with detectable synaptic connections (open symbols). The median values were 13.2 and 5.0 nA for motoneurons with and without detectable EMG activity, respectively. The median rheobase value for motoneurons with detectable connections is similar to the medians for normal experiments and saline controls (see above). For comparison, the median rheobase current value was 11.1 nA for 18 motoneurons that were studied in 1 animal 140 d after BTX administration and were all functionally connected with muscle fibers. Thus, motoneurons that possess what are likely to be very few unblocked synapses are associated with rheobase currents that appear to be normal, while motoneurons without detectable synapses are associated with axotomy-like rheobase values. This point is illustrated further by the fact that the motoneuron innervating the fibers whose EMG activity is shown in Figure 5*A* had a rheobase current of 27 nA. Such a rheobase current is only observed among normal MG motoneurons (Fleshman et al., 1981) and is almost 3 times greater than the highest value of rheobase current observed among MG motoneurons studied 21 d after peripheral axotomy (Pinter and Vanden Noven, 1989).

The association between the presence or absence of detectable synaptic connections with muscle fibers and the occurrence of a normal or axotomy-like distribution was also evident in the distributions of the sag ratio (Fig. 6*B*), input resistance (Fig. 6*C*), and membrane time constant (Fig. 6*D*). The differences between these distributions were all significant by the Mann–Whitney *U* test ($p < 0.01$). AHP distributions (not shown) also differed ($p = 0.02$). The shifts in these distributions indicate that the properties of "fast" MG motoneurons are substantially affected after BTX in a manner similar to that seen after axotomy (Gustafsson and Pinter, 1984b; Pinter and Vanden Noven, 1989). However, as was the case with motoneuron property distributions grouped according to whole-muscle force (see above), the differences between the "connected" and "unconnected" distributions of Figure 6 are not as pronounced as the differences between distributions obtained from axotomized and normal motoneurons (Pinter and Vanden Noven, 1989). No significant difference existed between the axonal conduction velocity distributions for "connected" and "unconnected" MG motoneurons (not shown; $p > 0.2$). The comparisons of motoneuron electrical properties among these various conditions are summarized in Table 2.

It should be noted that only 1 connected motoneuron was observed 14 d after BTX, while the remainder were obtained from experiments 21 d after BTX. This suggests the possibility of limited synapse reformation in the 14–21-d post-BTX interval. The possibility of such recovery is considered in detail in the Discussion. No significant differences existed in the properties of unconnected motoneurons sampled at 14 and 21 d after BTX (Mann–Whitney *U* test).

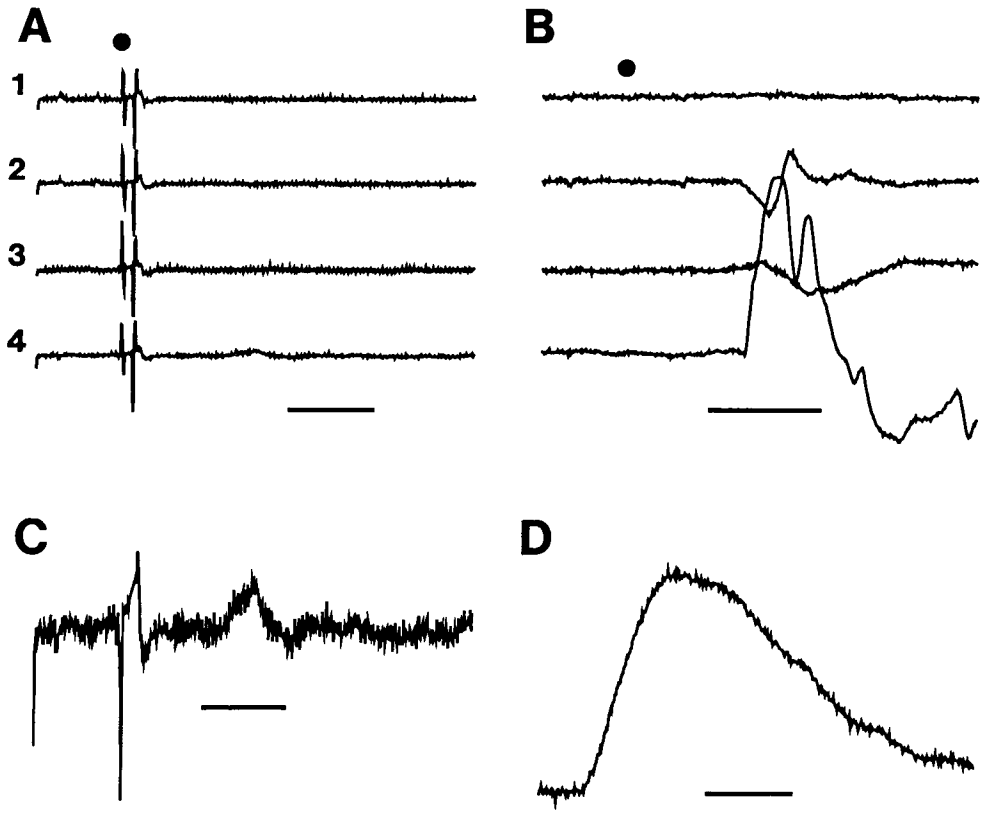

Figure 5. Averaged EMG records from 2 MG motoneurons innervating BTX-treated MG muscles. In *A* and *B*, each record is an average obtained from 1 of 4 implanted EMG electrode pairs after 100 trials of intracellular stimulation in each motoneuron. All records in *A* and *B* are shown at the same gain. The *solid dots* above the *top records* indicate the onset of the intracellular stimulation pulse. *A*, Data obtained from an MG motoneuron 21 d after BTX injection into the MG muscle. Note the presence of a small EMG signal in *record 4* only. *B*, Data obtained from an MG motoneuron 140 d after BTX injection into the MG muscle. Note the presence of EMG signals in *records 2–4*. Large-amplitude EMG records such as these were not observed 14–21 d following BTX injections. *C*, Record 4 in *A* shown at higher gain. *D*, Twitch force for motor unit shown in *B*, 140 d after BTX injection. The record is an average of 10 trials. The amplitude of this response is approximately 1 gm. Calibration: 4 msec, *A–C*; 20 msec, *D*.

Axon damage. It is possible that motor axons may have been damaged by the BTX injection procedure itself. If such damage included complete severance of motor axons, then axotomy-like properties would be expected among some motoneurons independent of BTX effects on neuromuscular transmission. We cannot exclude the occurrence of this problem among the data from BTX experiments. However, the results of the saline control experiments indicate that axonal injury sufficient to eliminate completely functional or physical contact between motoneurons and muscle fibers is an unlikely event. In these experiments, 18 motoneurons were recorded, and all possessed connections with muscle fibers detectable by motor unit force recording. It remains possible that the injections may partially axotomize motoneurons by injuring branches of motor axon terminal arbors. If such partial damage could provoke axotomy-like changes in motoneuron properties, one could reasonably expect to find evidence of such changes among "connected" motoneurons as well as the motoneurons from the saline control experiments. Both of these groups, however, display normal properties (see Table 2). It thus does not appear likely that such partial damage leads to axotomy-like changes. An indication of the absence of significant amounts of partial axotomy after saline injections is provided by a plot of the *unpotentiated* motor unit twitch force against the motor unit twitch time-to-peak (Fig. 7). Although the sample size is relatively small, the range of the unpotentiated twitch force is quite compatible with previously published data from the cat gastrocnemius (Burke, 1967).

Recurrent inhibition. There is evidence that a small amount

Table 2. Summary of motoneuron properties

	Unconnected	Connected	Saline	Normal	Axotomy
Rheobase, nA	5.0* (61)	13.2 (35)	11.5 (18)	11.0 (46)	3.4 (32)
Input resistance, mΩ	1.11* (57)	0.60 (29)	0.70 (18)	0.80 (44)	1.73 (32)
Sag ratio	0.94* (59)	0.73 (29)	0.71 (18)	0.80 (43)	0.99 (32)
Time constant, msec	6.71* (55)	4.50 (30)	5.00 (18)	5.20 (41)	7.72 (30)
AHP duration, msec	93* (58)	76 (34)	71 (18)	79 (42)	122 (30)
Conduction velocity, M/sec	92 (61)	87 (35)	87 (18)	91 (46)	68 (32)

This table displays median values for motoneuron properties. Values in parentheses indicate number of cells. Statistical tests (Mann–Whitney) in this table were limited to comparisons of connected and unconnected BTX data with data from saline control experiments. With the exception of conduction velocity, all other measured properties of unconnected BTX motoneurons differed significantly ($p < 0.01$, indicated by asterisks) from saline control data. There were no significant differences between connected and saline control data ($p > 0.10$). No differences existed between saline control experiments ($p > 0.10$). For comparison, normal and axotomized data from Pinter and Vanden Noven (1989) are also included. Axotomy data are from MG motoneurons axotomized 21 d earlier.

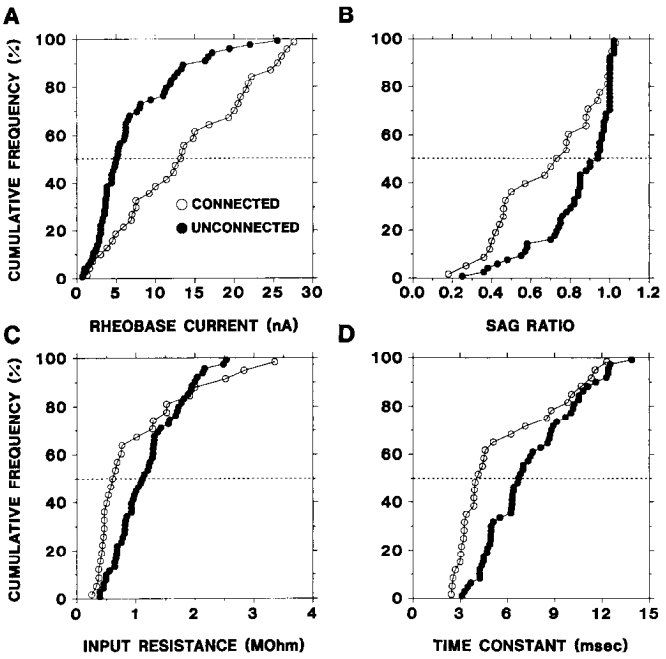

Figure 6. Cumulative histograms of MG motoneuron electrical properties grouped according to presence (connected, *open symbols*) or absence (unconnected, *solid symbols*) of detectable EMG signals following intracellular stimulation of MG motoneurons 14–21 d following BTX injections. *A*, Rheobase current. *B*, Sag ratio. *C*, Input resistance. *D*, Time constant.

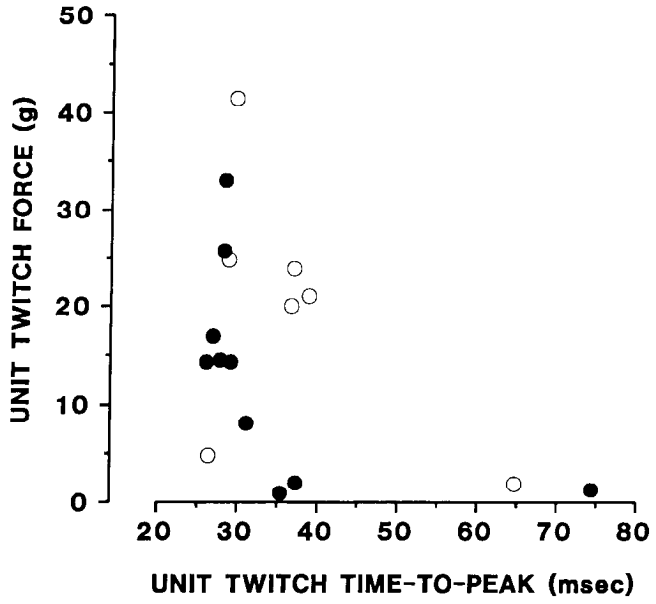

Figure 7. Plot of motor unit mechanical data from saline control experiments. Motor unit force (*ordinate*) represents the amplitude of the unpotentiated twitch contraction. Data from each saline experiment are represented separately as *open* and *solid symbols*.

of BTX or a part of the BTX molecule is retrogradely transported in muscle nerves (Haberman, 1974; Black and Dolly, 1986a). If such transport occurred in motor axons, it is conceivable that ACh release from motoneuron collaterals may also be affected. Available evidence indicates that recurrent inhibition from motoneurons whose motor terminals have been exposed to BTX is not blocked (Hagenah et al., 1977). In the present study, we recorded from a total of 16 antidromically identified LGS motoneurons in 5 of the 7 animals studied 14–21 d after BTX injections into the MG muscle. In each of the investigated LGS motoneurons, we checked for the presence of Renshaw inhibition following electrical stimulation of the MG nerve. In 14 out of 16 of these cells, recurrent IPSPs were observed. In 2 of these neurons, no recurrent IPSPs were observed. Because heteronymous Ia EPSPs from the MG nerve were also not observed in these 2 neurons, however, we suspect that these may have been type FF-L6 motoneurons in which heteronymous Ia EPSPs and recurrent IPSPs from the MG nerve can be quite small at resting membrane potential levels (Burke et al., 1976; Friedman et al., 1981). These results are consistent with previous findings of intact recurrent inhibition after BTX administration (Hagenah et al., 1977) and are compatible with evidence that the principle site of BTX action is the intramuscular motor terminal (Black and Dolly, 1986a,b).

Discussion

The results show that the presence of effective neuromuscular synaptic transmission at what is likely to be a nominal number of motor terminals is associated with normal motoneuron electrical properties. Thus, these motoneuron properties remain normal despite the blockade of neuromuscular transmission by BTX at most motor terminals. This scale of functional interaction is apparently sufficient to prevent the appearance of axotomy-like changes in the measured properties, because such changes seem to appear in motoneurons only when release of ACh from motor terminals is not sufficient to evoke detectable muscle fiber EMG activity. These results confirm previous indications that factors linked with effective neuromuscular transmission are involved in the regulation of motoneuron properties (Czeh et al., 1978) and add important new insight into the potency of the associated interactions between muscle and motoneurons.

How well does BTX mimic axotomy? Previous studies have shown that BTX treatment of muscle is associated with detachment of synaptic boutons from the motoneuron surface (Sumner, 1977), retraction of motoneuron dendrites (Sumner and Watson, 1971), and alterations of motoneuron metabolism (Watson, 1969). All of these phenomena are observed after axotomy. Recent evidence has shown that BTX is also associated with an axotomy-like somatofugal atrophy of motor axons, which is related to a decline in the expression of neurofilament protein (Hoffman et al., 1988). Our findings now extend this list to include axotomy-like changes of motoneuron electrical properties. It is important to note, however, that though the general pattern of axotomy-like change is present in our results, the extent of change in motoneuron electrical properties after BTX is less pronounced than after axotomy itself (see Table 2). Part of this quantitative discrepancy may be related to the possible failure of our EMG averaging technique to detect suprathreshold muscle activity in all cases. This would lead to the inclusion of "connected" motoneurons in the "unconnected" category and serve to disguise the true extent of difference between these groups. It seems unlikely that this can be the total explanation, however, because the somatofugal axonal atrophy observed after BTX is also less robust than after axotomy (T. Crawford, personal communication). The extent of this atrophy also helps to explain why conduction velocity changes were not observed presently; such atrophy must occur along a considerable portion

of an axon before it can manifest itself as a change of conduction velocity using our measurement methods. Overall, it seems reasonable to propose that BTX produces a qualitatively similar but somewhat attenuated version of the effects of axotomy.

Possible mechanisms. Because there is evidence that BTX or a fragment of the BTX molecule may be retrogradely transported in motor axons as far as the spinal cord (Haberman, 1974), the possibility exists that the axotomy-like effects we have observed arise because BTX directly affects motoneuron properties. While this possibility cannot be excluded, we believe that it is unlikely. First, any BTX that reaches the proximal part of the motor axon is either not sufficient for or not capable of blocking ACh release from recurrent collaterals (see Results; Hagenah et al., 1977). Second, it would be difficult to explain, on this basis, the presence of normal properties among motoneurons in which BTX has blocked ACh release from the majority of motor terminals.

Another possible view of these results is that the axotomy-like effects observed among motoneurons that cannot activate muscle fibers after BTX ("unconnected" motoneurons) reflect damage to motor axons or their terminal arbors caused by BTX and/or the injection procedure. The results from saline control experiments indicate that axonal damage caused by the injection procedure does not occur very frequently. There also exists extensive evidence that BTX itself is not associated with axonal or terminal damage that might elicit the changes of axotomy (for review, see Simpson, 1989). Thesleff (1960) observed, for example, that the ultrastructural appearance of motor axon terminals in the cat tenuissimus muscle was normal as long as 30 d after BTX injections, even though complete paralysis persisted. Other available evidence indicates that axonal transport and at least bulk-phase endocytosis at motor terminals remain intact after BTX (Pestronk et al., 1976; Kristensson and Olsson, 1978). Recently, Reiness and Lichtman (1988) have observed that the original but defunct motor terminals eventually retract from the original motor end plates in mice after BTX. This event appears to be associated with the formation of new synaptic contacts by motor terminal sprouts that appear after BTX (Duchen and Strich, 1968; Holland and Brown, 1981) and thus signifies the initiation of motor function recovery. In the interval between the initial paralysis and this retraction, however, motor axons and terminals appear to remain intact (J. Lichtman, personal communication), consistent with previous evidence that BTX does not directly damage axons or motor terminals.

Alternatively, it may be that differences in the level of terminal sprouting between motoneurons capable of activating a nominal number of muscle fibers and motoneurons not capable of this after BTX may contribute to the observed differences in electrical properties. It is well known that completely intact motor terminal arbors can display robust sprouting (Brown, 1984). There is thus no reason to suppose that the existence of a relatively small number of unblocked motor terminals could impose a differential limit on the extent of terminal sprouting that may have occurred among "connected" versus "unconnected" motoneurons in the present study. There is, however, evidence that the terminals of "slow" motoneurons may sprout more robustly than those of "fast" motoneurons after BTX (Duchen, 1970; Tonge, 1974; Brown et al., 1980). If this was the case in the present experiments, then it is unlikely that terminal sprouting per se makes any contribution to either the retention of normal motoneuron properties or the appearance of axotomy-like changes that we have observed. Among "unconnected" motoneurons, the shifts of median values of motoneuron properties to values more characteristic of "slow" motoneurons indicate that the properties of "fast" motoneurons are, in fact, altered despite the possibility that their terminals may sprout the least under these circumstances. Although the resolution of this question must await direct evidence, we currently do not favor the notion that terminal sprouting has played a role in determining the present results.

The known formation of new synaptic contacts secondary to terminal sprouting after BTX does, however, provide the possibility that the normal properties associated with motoneurons capable of activating muscle fibers are the result of such recovery. This seems unlikely for 3 reasons. First, previous work indicates that the cat hind limb muscle can remain fully paralyzed after large doses of BTX for at least 27 d (Thesleff, 1960). This suggests that cat terminals may sprout less readily after BTX than rat or mouse motor terminals (Brown, 1984), where recovery of nerve-evoked muscle force can begin as early as 10–12 d after BTX doses that are apparently as effective in causing paralysis (Tonge, 1974; Brown et al., 1980). Second, in preliminary work with the rat MG muscle, we found that 8 out of 15 animals showed small amounts of EMG activity following nerve stimulation 3–5 d following intramuscular injections of BTX at doses proportional to the doses used in this study (M. J. Pinter, S. Vanden Noven, D. Muccio, and N. L. Wallace, unpublished observations). This indicates that some motor terminals can escape blockade, at least with the BTX doses and injection procedure used in this study. Third, the first signs of recovery of the normal range of MG motoneuron properties in self-reinnervation studies appears to take at least a month after motor unit forces can be first detected (Foehring et al., 1986). This suggests that recovery of the normal range of properties is not associated with initial synapse formation and is likely to take *more* than a month following initial synapse formation with denervated muscle. In contrast to this, the full range of normal properties was observed 14–21 d after BTX among MG motoneurons capable of activating muscle fibers (Fig. 6). It thus seems more likely that the unblocked motor terminals detected in this study escaped the effects of BTX, and that the association of normal properties with "connected" motoneurons reflects a "sparing" phenomenon. On the basis of these arguments, we also believe that motoneuron properties were sampled within the interval between initial paralysis and the onset of new neuromuscular synapse formation, that is, before retraction of the original motor terminals.

At present, we can only speculate about the identity of other mechanisms that may account for our results. The absence of axotomy-like changes among "connected" motoneurons implies that the limited amount of associated neuromuscular transmission provides access to factors that are needed to maintain normal expression of motoneuron electrical properties. Trophic substances available from muscle are suspected of playing an important role in determining embryonic motoneuron survival (Oppenheim and Haverkamp, 1988), and it is conceivable that such substances may function in the adult to regulate or maintain motoneuron electrical and morphological properties (Czeh et al., 1978; Gallego et al., 1979; Foehring et al., 1987; see also Purves, 1986). Current beliefs are that such factors function in limited amounts (Barde, 1988), and such efficacy could help explain how greatly reduced levels of neuromuscular transmission could be associated with normal motoneuron properties. The available evidence is not sufficient to specify what steps in the chain of events that constitutes effective neuromuscular

transmission might be critical for access to such substances from muscle. The results of Gallego et al. (1979) imply that muscle contractile activity may not be necessary for the normal maintenance of at least some motoneuron properties, and that the metabolic state of the muscle (as indicated by its mass) may be of primary importance. Our findings of normal properties among "connected" motoneurons are not inconsistent with these possibilities, but do not distinquish between the effects of muscle fiber contractile activity and metabolic state.

It seems possible that the mechanism responsible for preventing changes among "connected" motoneurons is also responsible for limiting the extent of axotomy-like changes among "unconnected" motoneurons after BTX. One basis for this could be release of ACh itself in amounts not sufficient to activate muscle fibers, because BTX apparently does not block spontaneous, nonquantal release of ACh (Stanley and Drachman, 1983) or completely eliminate spontaneous quantal ACh release (Cull-Candy et al., 1976; Pestronk et al., 1976). However, Czeh et al. (1978) found a pronounced change of AHP duration among cat soleus motoneurons following 8 d of TTX blockade of motor nerve action potentials, and available evidence indicates that such blockade is not associated with changes of spontaneous ACh release (Drachman et al., 1982). Thus, the presence of normal levels of spontaneous release is apparently not sufficient to prevent changes in at least some motoneuron properties following elimination of normal neuromuscular transmission by TTX. This makes it seem less likely that the subnormal spontaneous ACh release associated with BTX could be involved in limiting the extent of axotomy-like changes among "unconnected" motoneurons. A comparison of the effects of BTX and of chronic postsynaptic blockade of neuromuscular transmission on motoneuron electrical properties could help in deciding the role of blocked ACh release itself or, more generally, failure of exocytosis in these changes of motoneuron properties.

An alternative possibility is that spontaneous activity of muscle fibers themselves, in the form of fibrillations (Josefsson and Thesleff, 1961; Purves and Sakmann, 1974), somehow limits the extent of axotomy-like changes among motoneurons that cannot activate muscle fibers by suprathreshold release of ACh after BTX. This would be more consistent with notions that muscle fiber activity or the presumably increased level of muscle metabolism allows the release of a factor(s) that is required by motoneurons for the expression of normal electrical properties (Czeh et al., 1978; Gallego et al., 1979).

More work will be needed to distinguish between these alternatives. The role of muscle fiber type in the specification of motoneuron properties will also need to be considered, because recent evidence indicates that muscle fiber type may influence the extent of recovery of motoneuron properties after axotomy (Foehring et al., 1987). On the basis of our results, however, it seems possible to speculate that the initial effects of axotomy itself on at least motoneuron electrical properties may be based specifically on the loss of factors that function successfully in limited amounts and are normally acquired by motoneurons at the neuromuscular junction as a result of effective synaptic transmission.

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