Prevention of Muscle Disuse Atrophy by Low-Frequency Electrical Stimulation in Rats

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***Abstract*—When muscles lose neural drive, they atrophy rapidly. Neuromuscular electrical stimulation (NMS) has been used in attempts to prevent or reverse the atrophy, but optimal stimulation programs and parameters are not well defined. In this study, we investigated the effects of four different stimulation patterns on disuse atrophy produced in the tibialis anterior, lateral gastrocnemius, and soleus muscles of rats paralyzed with tetrodotoxin for seven days. Stimulation paradigms differed from one another by their stimulation frequency (2 or 10 pulses/s) and by their stimulation period (2 or 10 h a day). Results showed that stimulation with 2 pulses/s, paradigms were more effective at preventing disuse muscle atrophy than higher-frequency stimulation. The most marked difference was in the slow soleus muscle, which had only 10% mean atrophy when stimulated at 2 pulses/s for 10 h, compared to 26% atrophy when stimulated at 10 pulses/s for either 2 or 10 h and 32% atrophy in unstimulated, paralyzed controls. The level of atrophic change was not correlated with the levels of serum creatine kinase, used as an index of muscle damage. Results suggest that remediation of disuse atrophy may be accomplished using unphysiologically low rates of motor-unit activation despite the relatively low force produced by such unfused contractions. This may have significant implications for the design of therapies for muscle paralysis consequent to upper-motoneuron lesions.**

***Index Terms*—Atrophy, disuse, electrical stimulation, rats.**

I. Introduction

DISUSE ATROPHY occurs in many neurological disorders (e.g., stroke and spinal-cord injury) when a loss of descending neural drive results in paralysis or paresis of muscles. Not only is disuse atrophy a pathology in itself, often requiring long-term rehabilitation, but it also contributes to other morbidities, such as shoulder subluxation and deep-vein thrombosis. Neuromuscular electrical stimulation (NMS) has been used to reverse the changes associated with the loss of voluntary muscular control. However, such treatments have been handicapped by a relatively poor understanding of optimal therapeutic parameters for reversing muscle atrophy and building strength.

To provide more insight into these relationships, much attention has been directed toward experiments in animals where the effects of different stimulation paradigms can be studied more systematically. Work performed on intact animals has suggested that the frequency and period of stimulation may affect the subsequent anatomical and functional features of muscle. For example, Salmons *et al.* [1] examined the progress of muscle changes when normal rabbit tibialis anterior muscles were stimulated continuously using pulse trains with different frequencies. Stimulation at 10 Hz, 24 h/day, was found to slow this fast muscle in only a few weeks. The transformation was characterized by an increased fatigue resistance, increased time to twitch, decreased force production and decreased size of the muscle. The physiological changes were associated with corresponding histological and phenotypic changes in myosin isozymes by week six of stimulation [1]. Similar results were obtained in rabbit tibialis anterior by using a frequency of 10 Hz, 8 h/day for seven weeks [2]. In contrast, stimulation with pulse trains at a lower frequency of 2.5 Hz had different effects. The muscle was faster and stronger than its counterpart stimulated at 10 Hz, albeit with a better capability to resist fatigue than a normal muscle. As well, histological examination showed that most fibers had staining profiles characteristic of type IIa fibers rather than type IIb fibers typical for this muscle under normal conditions [3], [4]. Results such as these emphasize the potential importance of identifying appropriate stimulation parameters if muscles are to be stimulated with electronic pulse-generating devices.

The electrical stimulation described was applied in addition to whatever muscle activation the animals produced during their daily activities. In muscles in which normal neural drive is lost, the situation becomes more complex. Changes due to the electrical stimulation are superimposed on muscles that have undergone disuse atrophy. Electrical stimulation may even prevent or reverse disuse atrophy and reduce the typical shift of muscle fibers toward faster, less oxidative phenotypes. Thus, the effectiveness of externally imposed stimulation is judged typically by the ability of stimulation to maintain or return muscles to a more normal state, rather than to convert them to some other form.

The most common experimental model to study the remediation of disuse atrophy has been the hemispinalized cat, in which stimulation has been applied to selected muscles for weeks or months. Kernell *et al.* [5] examined the effects of stimulating muscles with protocols consisting of 10- and 100-Hz trains of 10 pulses delivered every few seconds continuously for four and eight weeks. In all cases, this stimulation was found to induce fiber transformation toward type I profiles with small diameters and a decreased muscle capability to produce tetanic tension [5]. The loss of tetanic tension and fiber size was much smaller when

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a combination of 10 and 100 Hz were used [5]. Eerbeek *et al.* [6] also found that stimulus frequency and duration were important variables. They found that eight weeks of continuous stimulation at either 5 (with no pauses) or 10 Hz, 20 or 40 Hz (applied 1 s on, 1 s off) caused a marked prolongation of contraction speeds in peroneus longus. However, muscles stimulated using lower frequencies produced weaker maximal tetanic contractions than those stimulated with high frequencies. Gordon *et al.* [7] found that medial gastrocnemius muscles in hemispinalized cats produced less twitch and tetanic force when stimulated with 100- or 20-Hz regimens (applied 0.25/4.75 s on/off and 2.5/2.5 s on/off, respectively) for 24 h/day than with the same 20 Hz paradigm for only 3 h/day. The muscles stimulated at 20 Hz continuously were slower than those stimulated at the same frequency for only 3 h/day.

Experiments on hemispinalized cats are time- and resource-intensive. Furthermore, muscles on the affected side exhibit little atrophic change in the absence of electrical stimulation [5], so that interventions typically change muscle properties rather than remediate atrophy. An alternative approach that produces greater atrophic change involves the use of drug delivery systems to block the sodium channels of muscle nerves using tetrodotoxin. This approach can be applied easily in smaller animals, such as rodents, and produces a large and consistent level of muscle atrophy [8]–[11]. Tetrodotoxin (TTX), because it binds to the sodium channels of the axons, prevents the action potentials from reaching the motor endplate. The motoneurons and endplates remain intact and undamaged, axonal transport appears to be relatively normal and the blockade is complete but reversible after the source of TTX is removed [12]. Since the nerve is still present but quiet, this is similar to acute spinal-cord injury (below the level of the injury) or stroke. Electrical stimulation can then be applied to the nerves below the level of TTX application to investigate the efficacy of electrical stimulation to remediate the atrophic change. The feasibility of this approach has been demonstrated by Michel *et al.* [10] who showed that electrical stimulation (10 Hz for an hour, interrupted every 15 min for a 1-s train at 100 Hz, twice a day) for 7 days effectively reduced the atrophy that would normally follow TTX-induced paralysis of the rat soleus muscle. However, such experiments in rats have been hampered by difficulties in applying the electrical stimulation efficiently. In order to stimulate muscles in the experiments by Michel *et al.*, it was necessary to anesthetize the rats for each of the twice-daily stimulation periods [10], [12]. The regular imposition of anesthesia may produce stress-linked physiological changes, such as variation in the levels of circulating hormones, which may affect the physiological state of the muscles under study.

In the present work, we have investigated the usefulness of a the BION novel, inductively-powered and -controlled microstimulator for the chronic electrical stimulation of TTX-paralyzed muscles. These microstimulators are useful experimental tools because they can be programed to deliver electrical pulses in trains whose current, pulsewidth, frequency, and period can be controlled externally. Further, the microstimulators are sufficiently small (16 × 2 mm) for implantation into rodent limbs. In a previous study, we have established that TTX blockade in freely moving rats produces a predictable level of atrophy whose baseline features could be documented [11]. In this study, we have attempted to evaluate the course of development of this atrophy when three muscles with different roles and fiber-type compositions were stimulated electrically by varying the frequency and period of the stimulation. Our focus was on early-stage changes occurring before alterations in the enzyme profiles of different fiber types became evident. We found that atrophy was reduced most effectively in all of these muscles by low frequencies of stimulation that evoked unfused muscle contractions. These results may provide guidance for the application of electrical stimulation to clinical conditions.

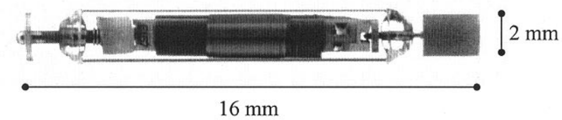

Fig. 1. Photograph showing the dimensions and physical features of the microstimulators.

II. Method

A. Surgical Implantation of Microstimulators and TTX Cuffs

Female Sprague–Dawley rats (Charles River, Toronto, ON, Canada) weighing between 200 and 250 g were housed in groups of 2–4, provided with rat chow and water ad libitum, and kept on a 12 h light/dark cycle. Each rat was anesthetized with ketamine (70 mg/kg body weight) and xylazine (5 mg/kg body weight) intraperitonally; additional doses of ketamine were administered as needed. Two small incisions, one along the tibia, and one on the posterior part of the ankle, were made on the lateral side of the left ankle of each animal. Space was freed between the tibia and the tibialis anterior (TA) muscle on the dorsal aspect, and between the lateral gastrocnemius (LG) and soleus on the ventral aspect of the limb. A single microstimulator was inserted in each location in animals in which stimulation was to be carried out (Fig. 1). Microstimulators were not implanted in control (nonstimulated) animals. An incision was then made on the lateral side of the left thigh of each animal and the biceps femoris muscle was retracted to expose the sciatic nerve. The sciatic nerve was freed from surrounding tissues and implanted with a nerve cuff connected to an osmotically driven drug-delivery system. To produce these implanted systems, mini-osmotic pumps (Alza Corp., Palo Alto, CA) were filled with 100-μl TTX solution (350 μg/ml in normal saline) (Sigma-Aldrich, Oakville, ON, Canada) and attached to a tube and nerve cuff, made in-house as a modification of the design used by Michel and Gardiner [9]. The tube and cuff system was made from Silastic tubing (Dow Corning, tube: 0.025″ID × 0.047″OD; cuff: 0.078″ID × 0.125″OD). The pump delivered the TTX solution constantly to the left sciatic nerve of the rat via the cuff at a rate of 0.5 μl/hr for seven days. Four sutures (3-0 silk) were used to close the nerve cuff. The TTX-containing pump was passed subcutaneously to a second incision made between the scapulae of the animal's back and positioned just caudal to the incision (Fig. 2). Muscle layers and skin openings were reapproximated with sutures. Lidocaine cream was applied

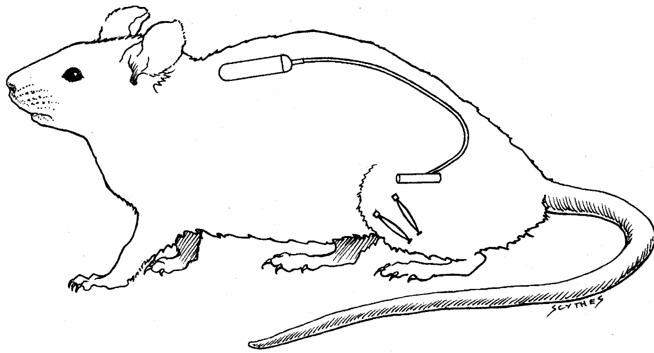

Fig. 2. Line drawing to schematically illustrate the location of implanted devices. The two microstimulators were placed on the dorsal and ventral aspects of the limb alongside TA and soleus, respectively.

around the skin sutures immediately following surgery and for the next 2–3 days to minimize sensations that might elicit grooming of the sites. Paralysis of the left ankle muscles developed within 24 h after surgery. The presence of paralysis was evaluated daily in all rats by the loss of toe-spreading and pinch reflexes [10], [13]. The paralysis was found to be absent in fewer than 5% of all rats. Self-mutilation of the foot and toes was rare. Bandages were used to cover the foot and toes for a few days if evidence was found that rats were attempting to chew their limb. Further, such behavior was not found to occur in later animals that were raised in groups rather than singly.

B. Stimulation

Microstimulators rely on an externally-placed inductive coil to provide power and control signals. A large inductive coil was constructed into which could be slid a Plexiglas cage of dimensions 17 cm wide $\times$ 36 cm long $\times$ 9 cm high. Food was placed at the bottom at the cage, and a water bottle was provided for the rats contained inside. The animals were in this cage for 1–10 h at a time, in accordance with the stimulation paradigm.

The microstimulator used in this series of experiments was made up of a small cylindrical glass capsule (diameter: 2 mm) that contained a receiving antenna coil and a miniature circuit board and integrated circuit chip. Stimulating electrodes were sealed hermetically at each end of the glass capsule. The cathodic electrode was made up of tantalum, while the anodic electrode was a thin iridium disk (Fig. 1) [14].

The rats were divided randomly into five groups: a control group (TTX paralysis only; no stimulation; $N = 17$) and four stimulated groups, as shown in Table I. Stimulation for all groups was done at supramaximal levels, observed qualitatively when stimulation was increased, but a plateau in the response (movement) was observed. Previous experiments have demonstrated the biocompatibility of the microstimulators themselves under both active and passive conditions [17], so no further controls were undertaken. In two groups, stimulation was carried out in two 1-h sessions spaced by at least 8 h. In the two remaining groups, 10 h of stimulation were applied to one half of the rats in each group during the day and to the other half during the night. No differences could be detected between rats stimulated during the day or night. Stimulation at 2 pulses/s resulted in twitch contractions throughout the stimulation period. Stimuli at 10 pulses/s produced a stronger unfused muscle contraction. In the group stimulated for 2 h a day at 10 pulses/s, high-frequency (50 Hz) trains of 1 s were applied every 15 min, to match approximately the protocol used by Michel and associates [10]. No high-frequency stimulation episodes were applied during other paradigms. In rats stimulated using the 10 pulses/s regimen for 10 h, stimulation was delivered intermittently (1 s ON, 1 s OFF) to protect blood flow to the muscles (Table I). Muscles were observed for the duration of the stimulus period to ensure that contractions continued without gross fatigue. Testing was done regularly during the seven days of stimulation to ensure that microstimulators were functioning well.

TABLE I
STIMULATION PARADIGMS USED IN STIMULATED ANIMALS

Frequency	Duration	N	Time period	Delivery method	# pulses
2 pps	2 hrs	7	2 x 1 hr	continuous	14 400
	10 hrs	10	1 x 10 hrs	continuous	72 000
10 pps	2 hrs	9	2 x 1 hr	continuous + 1 sec at 50 pps every 15 minutes	72 400
	10 hrs	8	1 x 10 hrs	1 sec on/1 sec off	180 000

A potential concern when using inductively-powered devices such as microstimulators is their sensitivity to orientation with respect to the field generated by the inductive coil to which they are coupled. Microstimulators typically maintain their inductive linkage as long as they are aligned $\pm$ 70° with respect to the long axis of the field. However, when devices become oriented perpendicularly in the field, communication can fail and stimulation can stop. The animals showed no propensity to align themselves preferentially so as to receive or avoid stimulation, so we have assumed that a small percentage of the intended stimulation may have been missed at random in all animals.

C. Muscle Weight and Histology

After seven days of paralysis, animals were anesthetized with sodium pentobarbital injected intraperitonally. The soleus, tibialis anterior and lateral gastrocnemius muscles were removed from both sides of the animal and weighed. In some instances, microstimulators were surrounded by connective tissue that lay close to the muscle. This tissue was removed as completely as possible from the muscle surface before the muscle was weighed. An intracardiac blood sample was taken for creatine kinase (CK) analysis. The rats were then immediately killed with an overdose of sodium pentobarbital injected in the heart. The belly of each muscle was mounted on a cryostat chuck in a recorded orientation. The muscle pieces were coated in talcum powder and frozen in liquid nitrogen. Blocks were frozen, cut into 12–16 μm sections and stained with hematoxylin and eosin (H&E) and for ATPase activity following alkaline preincubation (pH 10.4) [15], as described previously [11].

D. Data Analysis

Fibers were typed as I, IIa, and IIb according to their light, dark, and intermediate stain intensities. Relative contributions of different fiber types to the overall CSA of muscles were

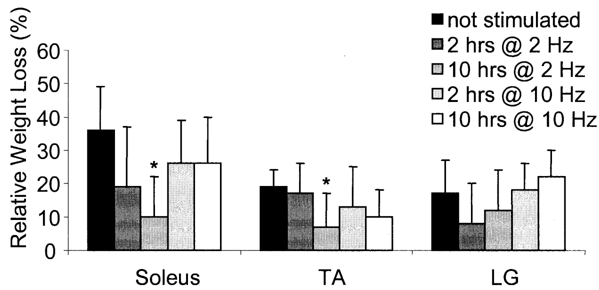

Fig. 3. Relative loss of muscle weight (compared to matched contralateral control muscles) when muscles are stimulated with different paradigms. Bars represent standard deviations. Asterisks represent significant difference ($p < 0.05$) from the weight loss of non-stimulated muscles.

estimated using methods described in the companion paper [11]. These proportions did not change significantly after seven days of paralysis, nor did they change after seven days of paralysis and stimulation. Fiber cross-sectional areas were measured using ImagePro software. Differences in muscle weight, fiber-type proportions, and fiber cross-sectional areas (CSAs) were evaluated using student T-tests with significance measured at $p < 0.05$ when data had a normal distribution. Data from groups of rats subjected to different stimulation treatments were compared using analyses of variance (ANOVAs) followed by student T-tests with significance measured at $p < 0.05$ when data had a normal distribution.

III. Results

Electrical stimulation of the paralyzed muscles evoked rhythmic muscle contractions and visible movement of the foot. Rats appeared undisturbed by these contractions, and continued to move in the cage, eat and sleep in a seemingly normal way throughout the stimulus period. Strong movements continued to be present even at the end of sessions using the longest durations and highest frequencies of stimulation. There was no obvious drop of contraction strength at the end of the seven days of stimulation. Occasionally, microstimulators did not seem to be activated in the coil; this resulted in a failure to reliably activate ten of the muscles under study. Data from these poorly stimulated muscles were excluded from the final analysis. The locations of all devices were checked at necropsy, and no evidence was found that devices moved from their sites of implantation.

A. Muscle Weights

Unstimulated Paralyzed Muscles: Paralysis for seven days resulted in a marked loss of muscle weight that has been described elsewhere [11]. TA lost 19±5% of its weight and LG lost 17 ± 10% compared to contralateral controls (Fig. 3). Paralyzed soleus muscles were significantly more atrophied than TA and LG, losing an average of 36 ± 13% of their weight compared to contralateral controls.

TABLE II
Relative Muscle Weight Loss (Expressed as a Percentage of Matched Contralateral Control Muscles) (Average ± Standard Deviation) Using Different Stimulation Paradigms

Frequency	Duration	Muscle	N	Weight loss
Unstimulated		Soleus	5	36 ± 13 %
		TA	15	19 ± 5 %
		LG	11	17 ± 10 %
2 pps	2 hrs	Soleus	4	19 ± 18 %
		TA	7	17 ± 9 %
		LG	6	8 ± 12 %
	10 hrs	Soleus	7	10 ± 12 %
		TA	8	7 ± 10 %
		LG	8	12 ± 12 %
10 pps	2 hrs	Soleus	7	26 ± 13 %
		TA	7	13 ± 12 %
		LG	7	18 ± 8 %
	10 hrs	Soleus	8	26 ± 14 %
		TA	5	10 ± 8 %
		LG	8	22 ± 8 %

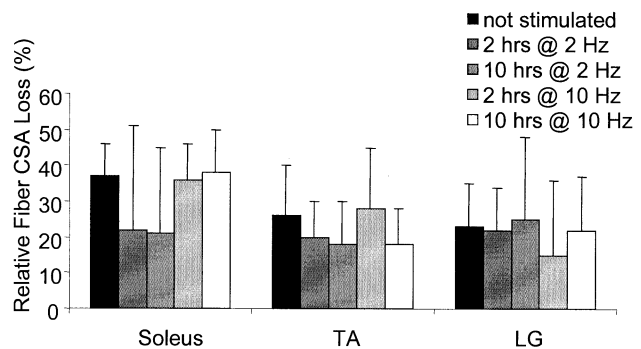

Fig. 4. Relative loss of fiber cross-sectional area (compared to matched contralateral control muscles) when muscles are stimulated with different paradigms. Bars represent standard deviations.

Effects of Electrical Stimulation: Differences were apparent in the degree of atrophy following electrical stimulation at different frequencies and durations. Electrical stimulation using 2 pulses/s stimulation was generally more effective than 10 pulses/s (Fig. 3). Stimulation using 2-pulses/s trains for 10 h/day decreased the average level of atrophy in soleus to 10 ± 12% (compared to 36 ± 13% for unstimulated muscles, $p < 0.005$). Atrophy in TA decreased significantly to 7 ± 10% (versus 19 ± 5%, $p < 0.01$). LG showed a more modest mean decrease that was not significant statistically [12 ± 12% versus 17 ± 10%, nonsignificant (NS)]. The same 2 pulses/s frequency applied for only 2 h was somewhat less efficacious in reducing atrophy. Nevertheless, it produced a significant decrease in the level of atrophy in soleus and LG. Results in TA differed little from control values (see Fig. 3 and Table II).

Electrical stimulation at 10 pulses/s was less effective than 2 pulses/s, whether it was applied for 2 or 10 h. Modest improvements were apparent in some muscles but average values were not significantly different from those of paralyzed muscles (see Fig. 3 and Table II).

B. CSAs

Changes in fiber CSAs followed the trends set by changes in muscle weight, but standard deviations were larger because of the large variations of fiber size depending on fiber type and intramuscular location. Thus, significant differences between treatment groups were not present. The degree of atrophy estimated from CSA was typically higher than that estimated from muscle weights (Fig. 4). Muscle tissue in all groups appeared

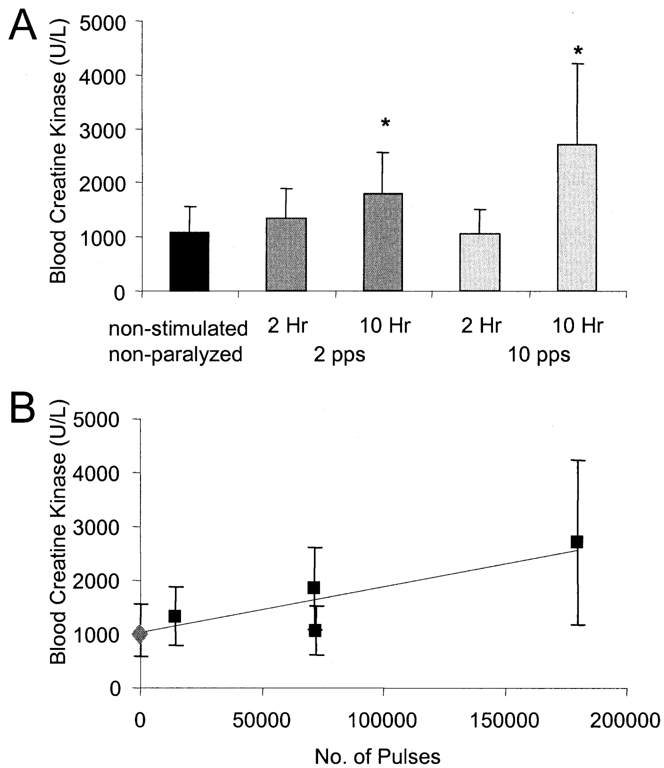

Fig. 5. Blood CK levels at the time of sacrifice in control and stimulated animals. (A) CK levels for different paradigms. $*$ indicated significant difference with controls (nonstimulated, nonparalyzed, $p < 0.05$. (B) Relationship between average CK levels and number of stimulation pulses received per muscle per day. ♦ indicates nonstimulated nonparalyzed animals.

TABLE III
CK LEVELS (MEAN $\pm$ STANDARD DEVIATION) FOR DIFFERENT STIMULATION PARADIGMS. $*$ INDICATES SIGNIFICANT DIFFERENCE FROM CONTROL ANIMALS ($p < 0.02$)

Frequency	Duration	# pulses	N	CK (U/L)
unstimulated		0	6	1070 ± 482
2 pps	2 hrs	14 400	7	1333 ± 551
	10 hrs	72 000	10	1803 ± 755 *
10 pps	2 hrs	72 400	8	1062 ± 456
	10 hrs	180 000	8	2695 ± 1526 *

normal and no increase was observed in the amount of connective tissue around the muscle fibers.

C. CK

The average creatine kinase (CK) level in nonparalyzed, nonstimulated rats was 1070 $\pm$ 482 units/liter (U/L). Stimulation tended to increase CK levels slightly, although differences were only significant in groups in which stimulation was applied for 10 h [see Table III and Fig. 5(a))]. CK levels were correlated ($r = 0.88$) with the number of stimulation pulses the rats received per day in each muscle [Fig. 5(b)]. CK levels were not, however, correlated with the degree of atrophy.

IV. DISCUSSION AND CONCLUSION

In this study, we have explored the effectiveness of electrical stimulation with different frequencies and periods on disuse atrophy induced by the complete motor blockade of rat hindlimb muscles. The rat is a species that has many advantages with respect to other experimental models in which studies such as these could be carried out. Rats are inexpensive and relatively homogeneous from a genetic perspective. Furthermore, much previous research on rat hindlimb muscles has furnished a comprehensive base of knowledge about normal anatomical and functional properties and responses to various experimental perturbations designed to produce disuse atrophy [16]–[19]. Chronic studies of responses to electrical stimulation have been handicapped by the technical problems of stimulating such small muscles repeatedly over time. The recent introduction of RF-controlled-and-powered microstimulators provides a new tool for studies of electrical stimulation in small animals. We have been able to confirm that electrical stimulation using this technology can produce a reduction in atrophy, and that the magnitude of this effect depends on the frequency and period of the stimulation paradigm. Perhaps surprisingly, the most efficacious paradigms employed frequencies of only 2 pulses/s. These frequencies are much lower than would be chosen by a strategy that sought to replicate the physiological firing frequencies of rat motoneurons, typically 30–80 pulses/s [20].

A. Technical Considerations

Our microstimulators, called BIONs (for BIOnic Neurons), are small enough (16 mm long $\times$ 2 mm diameter) to be amenable to implantation in small muscles such as those of rats. In past studies on rodents with TTX-induced paralysis, it was necessary to anaesthetize the rats daily in order to impose even brief, 1-h epochs of stimulation [10], [12]. Repeated bouts of anaesthesia are undesirable, not only because they are stressful for animals and can alter physiological and hormonal state, but also because they limit the practicality of longer epochs of stimulation such as the 10-h sessions used in the present work. When inductively-powered implants are used instead, alert animals can be housed without disturbance within the magnetic field of a large primary coil, and stimuli can be delivered while animals eat, sleep, and move normally [21], [22].

The BION microstimulators produce maximal stimulus pulses that are sufficiently strong to assure complete recruitment of the motor units in a rat muscle. Their pulse current and duration can also be controlled precisely to recruit any desired percentage of muscle activation. This feature is important in clinical trials that are currently underway [23]. In studies on cats, it has been shown that optimal recruitment of the muscle during stimulation is achieved when a BION is located near the motor point. Thus, they are placed close to the point where the whole muscle nerve enters the muscle, before it branches into separate neuromuscular compartments innervated by only a subset of the motoneurons. When a BION is located more distally within the same muscle, maximal recruitment is decreased [22]. In small animals such as rats, one BION can recruit more than one muscle if the cathodic end of the BION (the tantalum electrode) is located close to a common nerve branch [24]. In our laboratory, it has been shown that implanted BIONs do not migrate; this is probably due to the necked profile of the BION, which permits secure fixation and

encapsulation of the device by perimysial tissues [25]. Further, the stability of stimulus thresholds and the postmortem observations of device placement reinforced previous observations in cats that these devices do not generally migrate from their sites of implantation [21]. Thus, the impression was gained that devices could be used chronically not only for one week, as shown here, but for much longer periods in order to examine longer-term effects of muscle stimulation.

A second concern that arises when using inductively-powered microstimulators is the possibility that some of the intended stimulation may not actually be delivered, as a consequence of misalignment of the devices inside the primary coil. Although this problem was observed only infrequently, it introduces a small degree of unpredictability, estimated at about 20%. This might increase the variability of the results from different muscles in response to what would be otherwise considered to be an identical intervention. The implants and primary coils are currently undergoing further development to minimize or at least detect this potential problem. One approach is to provide an outgoing data link that would confirm when the commanded stimulation is actually delivered.

B. *Biological Sources of Variability*

In the present study, some variability was observed in the amount of atrophy present in all groups of muscles. This variability is perhaps not surprising because the rate of muscle atrophy is particularly rapid during the first week of paralysis. For example, the average loss of weight in soleus of 36% over seven days suggests a daily loss of approximately 5% during this period. Even small differences in rate from one animal to another could cause the muscles to differ in their weights and CSAs. Further, small differences in weight or fiber CSA may be introduced by even minor methodological inaccuracies. To analyze LG, for example, it is necessary to dissect away the attached MG; small differences in the placement of this division have been suggested previously to contribute to the typically larger variability of values for this muscle [11]. Occasional problems were also found when connective tissues enveloping an implanted device were closely adherent to an adjacent muscle. This problem was recognized as being potentially significant in soleus, whose very small weight could easily be biased by the presence of nonmuscular tissue.

Averaged fiber CSAs had larger amounts of variability than muscle weight, in part because the estimates of change in fiber CSAs depended on averaging measurements from many different muscle fibers of different types distributed in different muscle areas [11]. Atrophy as measured by loss in fiber CSA averaged 7% greater than atrophy by weight loss. Histological methods for frozen sections may introduce a small amount of shrinkage, but this should affect both atrophied muscles and their contralateral unparalyzed control muscles equally. One possible source of difference would be a tendency for atrophic muscles to shorten less than their normal controls during removal and handling associated with freezing, leaving their fibers longer and thinner. In order to eliminate such differences, it would be necessary to hold the muscles at a prescribed length during freezing, which is difficult to do after a muscle has been removed from the bone for weighing. In order to correct for such differences, it would be necessary to measure sarcomere length, which would require sectioning the muscle perpendicularly to the plane required for measuring CSA. Nevertheless, the trends identified by evaluating muscle weight were validated by identifying similar trends in fiber CSAs, suggesting that the results with either method were not greatly skewed by methodological problems.

Since we measured atrophy level by comparing the experimental muscle with its matched contralateral control, any errors will be magnified if they are in opposite directions on the two sides. In some previous studies, absolute weight measurements from the experimental sides were averaged and compared to averages on the contralateral control side without using a paired statistical strategy [9], [26]. Although such an approach might seem attractive, we were concerned that body and muscle sizes from one rat to the next might be quite variable. That source of variability can be eliminated by measuring atrophy of individual muscles relative to their contralateral controls.

C. *Effects of Different Stimulation Paradigms on Atrophy*

One central goal of the present study was to evaluate the efficacy of different frequencies and periods of stimulation on muscle atrophy. We were surprised to find that atrophy was remediated most effectively by stimulation frequencies of only 2 Hz compared to the more physiological frequencies of 10 Hz and above that have been typically used in previous studies on the effects of electrical stimulation [12], [27].

Two recent studies from one laboratory have examined the efficacy of electrical stimulation in TTX-paralyzed rats, using protocols in which frequency ranged between 10 and 100 pulses/s, or included a combination of 10 and 100 pulses/s epochs. Unfortunately, there are inconsistencies in results between these two studies, as well as some differences in methods with respect to our study. Michel *et al.* [10] reported that stimulation of soleus with parameters similar to one of the paradigms used in the present work (10 pulses/s for two 1-h periods, superimposed with 1-s bursts of high-frequency pulses) reduced the atrophy in soleus from values of 26% in control muscle to about 7% in stimulated muscles after six days of stimulation. Dunn and Michel [12] later compared the efficacy of four stimulation paradigms, including 10 pulses/s stimulation applied for an hour twice a day, 100 pulses/s stimulation applied either in 10 or 30 1-s epochs daily or a combined stimulation protocol matching that in the previous article. All four protocols provided some measure of protection from atrophy, but substantially less than reported in the original paper even for the matching paradigm, which produced only an 8% (NS) improvement in this later study. In soleus, the most effective paradigm used only 100 pulses/s stimulation (30 1-s epochs daily) and reduced the level of atrophy by about one-half [30% unstimulated, 16% stimulated (see Fig. 1 and [12])]. The other paradigms resulted in atrophy with values between 24% and 29% (NS compared to unstimulated controls). Our results in soleus using Dunn and Michel's paradigm of combined low and high frequency stimulation were similar to the insignificant

effects they reported in 1999, and were less effective than very low frequency stimulation (2 pulses/s) for 10 h a day.

In the same animals, Dunn and Michel [12] also examined the responses of plantaris, a fast ankle extensor and toe plantarflexor. Plantaris exhibited more sensitivity to the stimulation. Three of the four paradigms (10 pulses/s, 100 pulses/s, 10 1-s epochs/day, 10/100 pulses/s combined) remediated the atrophy by more than one-half with respect to nonstimulated muscles (22% nonstimulated versus 8%, 7%, and 10%, respectively). Our results in TA and LG showed less remediation of atrophy when a combined low-/high-frequency paradigm was used for 2-h/day (Fig. 3); no significant differences from nonstimulated paralyzed muscles could be demonstrated. Methodological differences may contribute to these discrepancies. In the experiments of Michel *et al.* [10], the animals were anesthetized and the knee and ankle (but not the toes) were restrained during stimulation to ensure isometric loaded contractions. Dunn and Michel [12] repeated the stimulation on rats whose limbs were free to move using one of the most effective strategies (100 pulses/s, 30 1 s epochs/day), and found much poorer results. In our studies, our animals were alert and generally had their legs tucked under their bodies, producing some degree of loading of the ankle extensor muscles, particularly for low frequencies of stimulation that produce little muscle force. We also stimulated flexors and extensor muscles with overlapping pulse trains to produce cocontractions. The dominance of the extensor muscles might have produced at least some ankle extension in some postures, resulting most often in eccentric contractions of TA and concentric but somewhat loaded contractions of soleus and LG.

Loading has been implicated as a factor in the application of electrical stimulation to reduce disuse atrophy in humans. Stein and coworkers have shown that identical stimulation paradigms in patients yielded different results depending on the loading conditions [29], [30]. They compared spinal cord injured subjects performing quadriceps muscle contractions against an isokinetic load or against gravity, in a sitting posture with the leg pendant, stimulation paradigms were identical (20 pulses/s stimulation in 5-s on/5-s off cycles, 1 h/day, 5 days a week, for 24 weeks). The group producing loaded contractions experienced an increase in torque of over 100%, while the group performing against gravity alone had a more modest torque increase of 50% [28], [29]. Even without overt loading, short trains of stimulation applied to individual muscles of humans would be expected to produce less muscle shortening than in rats because of the much larger inertia of their limbs.

Anesthesia may account for some differences in atrophic changes between the studies from Michel's laboratory [10], [12] and those reported here from nonanaesthetized animals. Anesthesia is known to trigger an increase in some hormone levels, including cortisol and growth hormone [31]. Growth hormone increases protein synthesis in muscles, but cortisol increases protein breakdown [32]. Without further study, it is difficult to know whether changes in hormone levels would change the rate of atrophy on the experimental side or even affect the mass of the muscle on the control side, which provides the denominator for assessing atrophy. The animals used in this study were female rats. It would also be interesting and valuable to repeat this experiment with male rats to determine whether the difference in hormones, mainly testosterone, would affect the response of muscle to electrical stimulation. Very little work has been done to identify whether males and females respond differently to neuromuscular stimulation.

The results observed in the present study are similar to those of Salmons and associates [1], [33], who found that stimulation at 2.5 pulses/s was more efficient at preserving muscle force than stimulation at 10 pulses/s in rats. Like our studies, those studies were performed without rigorous control over muscle loading. However, the studies by Salmons *et al.* [1], [33] were carried out in normal, nonparalyzed muscles in a different species and over a different survival period. Nevertheless, in both present and previous studies, better preservation of muscle force or CSA was achieved with a very low frequency of stimulation that caused the muscles only to twitch.

Recent studies of molecular mechanisms may provide some insight into potentially competing trophic mechanisms responsible for the selective effects of low frequency stimulation. Prolonged increases in basal levels of Ca++ appear to activate calcineurin, a phosphatase implicated in the up-regulation of slow-fiber-specific gene promoters [34], [35]. Stimulation at 10 pulses/s (producing partially fused contractions) for many hours each day might over time lead to the conversion of fast to slow fibers through sustained elevations in cytosolic Ca++ [34], [35]. Since slow fibers tend to be smaller in CSA, we would expect that such muscles would become smaller and less forceful, as has been observed. Calcineurin levels do not appear to be modified by the transient calcium spikes that might be generated by occasional, intermittent activation, but such stimulation still activates kinases that favor the maintenance of fast twitch fibers [36]. It may be that the 2 pulses/s stimulation is better able to keep basal calcium levels low, because it produces only intermittent twitching. This might then preserve the size and phenotypic distribution of the muscle fibers. Much further research is needed to understand the events that lead to fiber-type transformation and to fiber atrophy/hypertrophy, because these two types of change are not likely to be modified through a single or simple pathway. For example, disused muscle typically experiences transformation from slower to faster muscle-fiber composition, yet those fast fibers are markedly atrophic.

D. CK and Muscle Damage

Are some of the results reported here related to the degree of muscle damage produced by the different stimulation paradigms? In the past, some investigators have argued that the production of muscle-fiber damage is a necessary prerequisite for muscle fiber strengthening, while others find that it is detrimental [37]. CK plasma levels have been used as an index of muscle fiber damage in a number of studies where they have shown a correlation between CK and exercise duration, electrical stimulation frequency, and force increase during training [38], [39]. CK levels are known to correlate well with the amount of muscle soreness following strenuous and eccentric exercise [40], further suggesting a reasonable linkage to the amount of muscle damage. In the present studies, however, we

found little correlation between CK levels and the remediation of atrophy produced by a given stimulation protocol. The most effective paradigm to reduce muscle atrophy (10 h of stimulation a day at 2 pulses/s) was associated with intermediate levels of CK. CK levels seemed instead to be correlated more simply with the number of pulses delivered to the muscles [Fig. 5(b)]. This result is consistent with the results of Lexell and colleagues [41] who observed that that rabbit muscles stimulated at lower frequencies, resulting in fewer pulses, for the same period, had less muscle damage as determined from histological inspection than muscles stimulated at high frequencies. Child and associates [39] reported that higher CK levels were present after stimulating human muscles at 100 than at 20 Hz. It should be remembered, however, that CK elevation is a nonspecific indicator of muscle damage from virtually any mechanism. For example, Costill *et al.* [38] showed that CK levels increased with two exercise bouts of 30 s but did not increase if the same exercise was spread over ten bouts of 6 s each. The results imply that higher intensities of contraction elicited by high-frequency stimulation can be responsible for the damage seen in some situations. In our experiments, CK levels were measured only at the end of one week of stimulation. It would be interesting to know if the results would differ if we evaluated CK levels at an earlier point in the experiment, when acute damage to the fibers might be more apparent.

E. Clinical Implications

The results presented here have significant clinical implications. Typically, remediation of muscle atrophy using electrical stimulation in humans is carried out using stimulation frequencies of at least 10 pulses/s and often up to 50 pulses/s. High-frequency stimulation of loaded muscles for short periods results in high-force contractions that have been reported to increase muscle size. However, energetic, intensive exercise sessions are not appropriate for many paralyzed patients who may be older, disabled or osteoporotic and at risk of bone fractures from the application of high transient forces. The results presented here suggest that significant protection from atrophy may derive from a long-term program of low-frequency stimulation. Such a program of stimulation in human stroke survivors with shoulder subluxation has recently been shown to increase shoulder functionality and muscle strength [23]. As devices such as microstimulators become available for rehabilitative applications, it will be important to understand better the stimulation parameters that have the best likelihood to improve muscle strength and performance. This will require both animal studies in which conditions of paralysis are controlled rigorously, and human studies in which a full range of paretic and paralytic states can be treated for extended periods.

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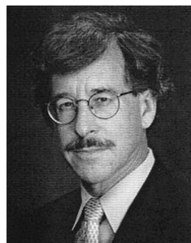

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