Effects of botulinum toxin A injection and exercise on the growth of juvenile rat gastrocnemius muscle

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Chen, Chen-Ming, N. Susan Stott, and Heather K. Smith. Effects of botulinum toxin A injection and exercise on the growth of juvenile rat gastrocnemius muscle. J Appl Physiol 93: 1437–1447, 2002. First published June 30, 2002; 10.1152/japplphysiol.00189.2002.—Botulinum toxin A (Btx) injections and supervised exercise are often used concurrently to treat calf muscle spasticity in children. This study has analyzed the early effects of Btx-induced paralysis and increased activity by voluntary wheel running on cell growth-related processes in juvenile rat gastrocnemius muscle. Btx injection at 29 days of age prevented the normal increases in wet mass (50%) and fiber cross-sectional area (34%) seen by 36 days of age in control rats. Btx-injected vs. contralateral muscles had 22% fewer myonuclei per fiber length but greater than twofold the number of MyoD-positive nuclei at 36 days of age. The accretion of 5-bromo-2′-deoxyuridine-labeled newly produced myonuclei did not differ between limbs. Voluntary exercise during the 7 days increased the mass (18%) and fiber size (23%) of Btx-injected and contralateral muscles but did not affect any other variable. Thus Btx injection and exercise had early effects on muscle and fiber size without consistently associated changes in myonuclear production or number. This suggests the presence of noncontractile activity-dependent, growth-promoting cytoplasmic events in juvenile muscle.

voluntary wheel running; satellite cells; MyoD; myonuclear number; fiber size

Botulinum toxin A (Btx) is a potent neurotoxin that induces a prolonged, but reversible, paralysis of skeletal muscle (for a recent review, see Ref. 48). Intramuscular injection of Btx is used in the clinical treatment of dystonia and the muscle spasticity seen in children with cerebral palsy (12, 30). The toxin diffuses locally, enters nerve endings, and, through proteolytic events, prevents the release of acetylcholine at the neuromuscular junction (10). Fiber self-reinnervation via neuronal sprouting begins soon after the injection, but functional activity of these multiple sprouts takes several weeks (11, 31). Thereafter, the activity of the parent terminal returns and the accessory sprouts retract, thus reverting to the original innervation (15, 48). During this time, there is a corresponding initially progressive, then persistent atrophy, followed by a gradual recovery of muscle and fiber size (18, 31). The gross anatomic and neurophysiological effects of the toxin on the muscle and the associated changes in contractile function and motor skills are well documented (22, 33, 44). However, the myocellular (myonuclear or cytoplasmic) events related to the observed changes in muscle fiber size have rarely been considered (18, 31). Furthermore, although the toxin is commonly used in the treatment of young children, its effects on cellular events contributing to postnatal muscle growth have yet to be determined.

The processes underlying muscle growth include the provision of additional myonuclear DNA by the activation and proliferation of a proportion of the endogenous myogenic (satellite cell) population and their fusion with the existing fibers (23, 50). This increase in myonuclear content may help to regulate fiber size via the availability of DNA for RNA transcription. Alternatively, the number of myonuclei may be adjusted in response to changes in muscle fiber size to maintain the coordination of the multinuclear protein expression (1). Regardless, the addition of new myonuclei contributes to postnatal maturational growth and adult muscle hypertrophy, as, in rat muscle, irradiation to prevent satellite cell mitotic activity severely compromises increases in myonuclear number, fiber size, and muscle mass (56, 58).

In normal adult rat muscle, a reduction in neuromuscular activity leads to a decrease in muscle fiber size and may also be associated with a loss of myonuclei (1). Conversely, increases in muscle activity or loading enhance the production and accretion of new myonuclei in concert with an increase in fiber size, thereby maintaining a constant myonucleus-to-cytoplasmic volume ratio, or myonuclear domain (1, 39). In rapidly growing young rats, normal weight-bearing activity is important for the proliferation of satellite cells and the accretion of new myonuclei, and a withdrawal of, or reduction in, neuromuscular activity compromises muscle growth (14, 51, 61). In children with cerebral palsy, physical exercise or increased voluntary activity is often used as adjunct therapy to assist muscle maturational development and motor function (7, 8750-7587/02 $5.00 Copyright © 2002 the American Physiological Society
The influence of increased activity and its possible interactions with the effects of the Btx on cellular events related to postnatal fiber growth within the muscle have not been addressed in either human or animal studies.

The injection of Btx causes a localized muscle paralysis without disruption of axonal transport or the integrity of synaptic contacts (or products of nerve degeneration and retraction), allowing trophic exchange to persist (18, 34). The paralysis of the injected gastrocnemius muscle does not prohibit voluntary use of the limb (35). Therefore, although the muscle is limited in the active tension it can produce, it is involved passively during locomotion and other activities. In rats, limb use can be altered by the provision of running wheels in the cages. Rats will run spontaneously and regularly, at speeds sufficient to recruit the ankle plantar flexor muscles (57). Therefore, with the use of these techniques, the combined effects of Btx-induced paralysis and increased muscle activity and thus the importance of neuromuscular transmission and active and/or passive muscle activity on cellular processes related to postnatal fiber growth of a fast ankle plantar flexor muscle can be investigated.

We hypothesized that prevention of neuromuscular activity by intramuscular Btx injection would compromise the production and accretion of myonuclei and fiber growth, whereas increased neuromuscular activity by voluntary exercise would enhance these processes, in juvenile muscle. We also anticipated that the same exercise would have positive effects on myonuclear production and fiber size in Btx-injected muscle, thereby demonstrating growth-regulating mechanisms independent of the active contraction of the muscle.

The aim of this study was, therefore, to determine the effects of Btx injection-induced paralysis and/or increased limb use by voluntary wheel running (hereafter referred to as exercise) on cellular processes related to postnatal growth in juvenile rat gastrocnemius muscle. Specifically, early changes in muscle mass, fiber size, the incidence of MyoD-positive myonuclei, new myonuclear production, and myonuclear number were investigated. Of particular interest were the effects of Btx on normal muscle growth and the effects of Btx-induced paralysis and concurrent daily exercise in the rapidly growing young muscle. Initial evaluations of the systemic effects of the Btx, i.e., on weight gain and voluntary exercise behavior, and of possible compensatory loading effects on the nonparalyzed limb were also made.

Our results showed that fiber size and myonuclear number are reduced 7 days after Btx injection, indicating an essential role for neuromuscular activity in sustaining these processes during growth. Exercise enhanced muscle and fiber size to the same extent in Btx-paralyzed and intact muscles, while not affecting the changes in myonuclear production or number, thereby suggesting a noncontractile activity-dependent pathway for growth-promoting cytoplasmic events in juvenile fast muscle.

### METHODS

#### Animals

Twenty-one male Wistar rats (29 days old) were used in this study. Rats were housed in a temperature-controlled room maintained on a 12:12-h light-dark cycle with food pellets and water available ad libitum. All procedures for animal care and treatment were approved by the University of Auckland Animal Ethics Committee.

Rats were allocated to one of five groups. Rats in the first group received only saline (sham) injections (procedures below) into both gastrocnemius muscles and were killed the next day (NoBtx-30d; n = 3). Rats in the second and third groups were injected with Btx in the right gastrocnemius (Btx) and saline in the left gastrocnemius (NoBtx) and were then housed in either a standard cage (NoEx; n = 6) or a cage with a running wheel (Ex; n = 6) for 7 days. The fourth and fifth groups of rats received saline injections in both gastrocnemius muscles and were housed as per NoEx (n = 3) or Ex (n = 3) for 7 days. These groups were used to test for possible systemic effects of the Btx by comparison of gains in body mass and voluntary exercise distances with those of the groups that received unilateral Btx injections. The wet mass of the muscles from these groups was also compared with that of the NoBtx contralateral muscles of Btx-injected rats to test for possible compensatory loading effects on the nonparalyzed limb. When rats received only saline injections, both gastrocnemius muscles were included in the group, resulting in six muscles per experimental treatment.

To determine the effects of Btx on normal muscle growth, we compared wet mass, fiber size, the incidence of MyoD-positive nuclei, and myonuclear number in the Btx and NoBtx muscles from rats 7 days after the injection (i.e., at 36 days of age, all NoEx) with those of the NoBtx-30d muscles.

To assess the effects of the Btx, the effects of Ex, and the combination of both Btx and Ex on growing muscle, we made comparisons between the gastrocnemius muscles of both limbs (Btx and NoBtx) and between these muscles from rats that exercised (Ex) and rats that did not have access to a running wheel (NoEx).

The 7-day experimental period was chosen to be of sufficient length for substantial muscle growth and provide a suitable time point for the assessment of the extent of satellite cell activation, replication, and fusion (62, 65).

#### Intramuscular Injections

Rats were anesthetized with a methoxyfluorane-oxygen mixture, and the gastrocnemius muscle of each limb was injected at two sites (medial and lateral heads) with either 10 μl of Btx solution or sterile saline, in accordance with their experimental grouping. The Btx (BOTOX, Allergan) was reconstituted before each use, diluted to a final concentration of 50 U/ml in sterile saline, resulting in a dose of 1 U (0.4 ng) toxin per rat (≈9 U/kg body mass). This dose is similar to that previously shown to be effective in inducing functional deficits after injection in adult rat gastrocnemius muscles (33, 35). It is also higher than the reported median effective muscle-weakening dose (6.2 U/kg; Refs. 3, 4) but well within the safety margin of this Btx preparation. The lethal dose at which 50% of mice die after hindlimb intramuscular injection is 81.4 U/kg body mass (4). The volume of the toxin solution relative to the size of the muscle (≈380 mg) was also large, enabling a wide diffusion of the toxin throughout the muscle (9, 63).

The dose, volume of vehicle, and method of administration of the toxin were established in preliminary trials to result in...
visual muscle paralysis by the subsequent day and cause only minor inhibition of locomotory ability and no obvious systemic changes, such as a reduction in the gain of body mass, within the subsequent week. In addition, the cross-sectional area of fibers from multiple regions across proximal, middle, and distal sections of the medial and lateral heads of the muscle have shown significant atrophy compared with noninjected contralateral muscles, indicating that adequate toxin is also delivered to affect the majority of fibers throughout the muscle (unpublished observations).

In Vivo Cumulative Labeling of Newly Produced Myonuclei

Continuous infusion of 5-bromo-2’-deoxyuridine (BrdU; Sigma Chemical, St. Louis, MO) was used for the identification of myonuclei produced in the gastrocnemius muscles during the 7 days after the intramuscular injections. Mini-osmotic pumps (ALZET 2ML1, Alza Scientific, Palo Alto, CA) filled with 2 ml of BrdU solution (10 mg/ml) were implanted in the still-anesthetized rats directly after the injections in all except the NoBtx-30d rats. The preprimed (4 h in sterile saline at 37°C) pumps were placed subcutaneously via a small incision made in the skin above the scapulae. Designed to deliver 10 μl of solution per hour over the implantation period, they were left in place until after the death of the animals 7 days later. The volume of the remaining contents of each pump was then aspirated and measured to confirm adequate delivery of the solution (1.93 ± 0.09 ml for all rats). This concentration of BrdU results in circulating levels that far exceed those of the endogenous thymidine that it replaces, and thus all replicating satellite cell nuclei and daughter nuclei that subsequently fused to become mature, postmitotic myonuclei should be labeled during the exposure period. This concentration of BrdU administered for 1–2 wk does not inhibit in vivo satellite cell proliferation or fusion (62).

After the injections and pump implantation, the rats were placed back in standard cages or in cages with a metered running wheel, according to their allocated experimental group. Running distances were recorded daily. Paralysis of the Btx-injected muscles was confirmed visually, the day after injections and before death, by using a five-point scale based on the toe spread reflex response (3, 4).

Tissue Collection and Processing

At death, rats were deeply anesthetized by an intraperitoneal injection of pentobarbital sodium (Somnotol, MTC Pharmaceuticals), and the gastrocnemius muscles of both limbs were excised and weighed. The medial head of the muscle was cut into proximal and distal portions, and each sample was coated in embedding medium (Tissue-Tek OCT, Miles, Naperville, IL), immersed in isopentane cooled by liquid nitrogen, and stored at −80°C until use. Transverse sections (10 μm thick) were cut from the distal muscle samples at −20°C, mounted serially onto adherence-coated slides, and stained for the identification of the myogenic regulatory (transcription) factor MyoD1. The proximal portion of the medial head of the muscle was processed to obtain individual fiber segments free of interstitial cells and adherent fibroblasts, by using procedures adapted from those described previously (54). Muscle fibers were thawed progressively over a 24-h period, mechanically isolated in a low-Ca2+ relaxing solution, air-dried onto slides, and stained for BrdU-labeled and non-BrdU-labeled myonuclei.

Immunocytochemistry

Muscle sections were stained for MyoD1 for the identification of activated and proliferating myogenic (satellite) cell nuclei (42, 60). Whereas activated and proliferating satellite cells specifically express MyoD, the factor can also be detected for a short time after satellite cell fusion and, therefore, in some myonuclei within the fiber (24, 43).

Cryosections were fixed in 1% paraformaldehyde in 0.1 M PBS for 15 min, washed in PBS, and quenched in 0.3% hydrogen peroxide in 0.05 M Tris-buffered saline for 10 min. After incubation with anti-MyoD (Mab 5.8A, 1:40; BD Biosciences, San Jose, CA) in Tris-buffered saline with 0.1% Tween 20 (TBST) for 2 h, they were washed in TBST for 10 min and incubated with a biotinylated sheep anti-mouse secondary antibody (Amersham Pharmacia Biotech, Auckland, New Zealand) diluted 1:100 in TBST for 30 min. An avidin-biotin peroxidase solution (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA) was applied for signal amplification for 30 min, and then the reaction was developed with a diaminobenzidine solution (Vector) for 15 min.

A commercially available (Roche Diagnostics) mouse monoclonal IgG1 primary antibody directed against BrdU was used to detect nuclei that had incorporated the BrdU into their DNA during the 7-day exposure period within the individual fiber segments (i.e., newly produced myonuclei). Muscle fibers were incubated sequentially with 0.03% hydrogen peroxide in PBS for 30 min, 10% normal horse serum (NHS) in PBS for 20 min, and the primary antibody, diluted 1:10 in the antibody kit-provided Tris buffer solution, for 35 min at 37°C. After reincubation with NHS, fibers were incubated with biotinylated anti-mouse IgG (Vector), diluted in 2% NHS in PBS for 35 min and then in avidin-biotin peroxidase for 30 min, and developed (30 min) with an aminoethylcarbazole substrate solution (Vector). Fibers were counterstained with Mayer’s hematoxylin for the identification of non-BrdU-labeled nuclei.

Quantification and Analysis

Muscle sections and individual fibers were analyzed by using a Nikon E600 bright-field microscope and imaging software (ImagePro Plus, Media Cybernetics, Silver Spring, MD). Images were obtained with a color charge-coupled device camera (SPOT, Diagnostic Instruments, Sterling Heights, MI). The number of BrdU-labeled and non-BrdU-labeled myonuclei were counted from 20 fiber segments (980 ± 220 total myonuclei) from each muscle. The fiber segments (1,368 μm long) selected for analysis were intact, free of surface cells and connective tissue, and presented an undistorted view for imaging. Sarcomere length was determined from the mean of three measurements of the length of 10 Z-bands in series along the fiber and used to normalize the length of each fiber segment to a standard sarcomere length of 2.7 μm. The number of BrdU-labeled, newly produced myonuclei and the total number of myonuclei (BrdU labeled and non-BrdU labeled) per millimeter fiber length were calculated from the above values.

Muscle fiber cross-sectional areas and the number of MyoD-positive nuclei were determined from a total of 200 fibers from three or four nonoverlapping images from across one transverse section from each muscle sample.

The medial gastrocnemius in adult rats is composed of ≤10% of “slow” or type I myosin heavy chain-containing fibers, with a mixture of “fast” or type II (predominantly IIb and IId/x) myosin heavy chain isoform-containing fibers providing the balance (16, 26). Fiber-type proportions vary regionally within the muscle; however, for our measurements...
All rats with running wheels ran by the second night and daily thereafter (Fig. 1). There was a large variation in the total distance run by all rats (range = 2,883–21,330 m) such that there was no significant difference in this variable between the Btx-Ex and the NoBtx-Ex rats ($P = 0.54$).

The relative wet mass of the saline-injected contralateral muscles of Btx-NoEx and Btx-Ex rats was not significantly different from that of muscles of rats injected with saline in both limbs but otherwise treated in the identical manner for 7 days. This indicates that there was little or minimal compensatory loading of the nonparalyzed contralateral limb, or it was at least insufficient to affect muscle growth during the time examined.

Because there were no apparent systemic or compensatory loading effects of the Btx injections, the saline-injected contralateral (NoBtx) muscles were used for comparisons with the Btx muscles for all subsequent analyses.

**Muscle Wet Mass**

The difference in wet mass between NoBtx-30d and the 36-day-old NoBtx-NoEx muscles (0.381 ± 0.109 vs. 0.571 ± 0.125 g; 49.9%) was in proportion to the ≈50% difference in the rats’ body mass. The mass of Btx-NoEx muscles (0.381 ± 0.109 g) was not significantly different from that of the NoBtx-30d muscles (0.384 ± 0.096 g) but was significantly less when expressed relative to the rats’ body mass ($P < 0.001$).

The wet mass relative to body mass of Btx (NoEx and Ex) muscles was significantly less than that of the contralateral NoBtx (NoEx and Ex) muscles ($P < 0.001$) (Fig. 2). Ex muscles were significantly heavier than NoEx muscles ($P = 0.01$). There was no interaction effect ($P = 0.34$) between the two variables; the
effect of the exercise on muscle mass was independent of the effect of the Btx and vice versa.

Fiber Cross-sectional Area

The 36-day-old NoBtx-NoEx muscles had a 34% greater mean fiber cross-sectional area than did NoBtx-30d muscles (Fig. 3A). In the Btx-NoEx muscles, mean fiber area was 19% smaller than in NoBtx-30d but did not reach statistical significance ($P = 0.20$).

The Btx muscles had significantly smaller mean fiber areas than the contralateral NoBtx muscles (Fig. 3A) (836 vs. 1,239 $\mu m^2$, SE of difference = 52.6, $P < 0.001$). Exercise resulted in a larger mean fiber cross-sectional area than in NoEx muscles (1,154 vs. 940 $\mu m^2$, SE of difference = 63.5, $P = 0.007$). There was no significant interaction effect between Ex and Btx on the mean fiber area ($P = 0.38$).

When the distributions of individual fiber cross-sectional areas, illustrated by fiber area-frequency histograms in Fig. 3B, were analyzed, those of the Btx-NoEx muscles were significantly smaller than those of NoBtx-30d rats, indicating fiber atrophy. There were also significant differences in individual fiber areas between NoBtx-NoEx and NoBtx-Ex, and between Btx-NoEx and Btx-Ex muscles (both $P < 0.001$).

MyoD-positive Nuclei

With normal growth (NoBtx-NoEx vs. NoBtx-30d), there was a decrease in the number of MyoD-positive nuclei ($P = 0.009$). The Btx increased the number of MyoD-positive nuclei over that seen in the NoBtx-30d muscles ($P = 0.006$) (Fig. 4).

The number of MyoD-positive nuclei was also significantly higher in Btx-injected than in the contralateral
NoBtx muscles ($P < 0.001$), whereas there was no significant effect of Ex on this variable nor an interaction effect between Btx and Ex (Fig. 5).

**New Myonuclear Production**

All groups demonstrated low mean numbers (0.4–0.8 per mm) and percentages (0.8–2.2% of myonuclei) of BrdU-labeled, newly produced myonuclei (Fig. 6). There were neither significant main effects of Btx or Ex, nor an interaction effect between Btx and Ex on the number (or percentage) of BrdU-positive myonuclei per millimeter fiber length (all $P > 0.2$).

**Myonuclei per Millimeter Fiber Length**

There was a nonsignificant 5–6% difference in the mean number of myonuclei per millimeter fiber length between the 36-day-old NoBtx muscles and the NoBtx-30d muscles ($P = 0.66$). However, in Btx-NoEx muscles, there were 19% fewer myonuclei per millimeter fiber length than in the NoBtx-30d muscles ($P = 0.03$).

In Btx-injected muscles, there were significantly fewer myonuclei per fiber length than in NoBtx muscles ($P = 0.002$), the magnitude of which was independent of Ex. There was no effect of Ex on the number of myonuclei per fiber length (Fig. 7).

**DISCUSSION**

Intramuscular Btx injection led to muscle fiber atrophy and a proportionate net myonuclear loss, despite an increased number of MyoD-positive nuclei, after 7 days. The net effect of these changes was to compromise the maturational growth of the muscle. Voluntary wheel running enhanced muscle growth via increases in muscle fiber size but did not affect the number of MyoD-positive nuclei, new myonuclear production, or myonuclear number per fiber length. Importantly, the effects of Ex were independent of those of the Btx (and vice versa) for all variables. The combination of Btx and Ex thus resulted in an attenuation of the reduction in fiber size but did not affect the increase in MyoD-positive nuclei or the reduction in myonuclear number in the Btx-paralyzed muscle.

**Normal Growth of Juvenile Gastrocnemius Muscle**

**Muscle wet mass and fiber size.** The 50% difference in muscle wet mass between 30 and 36 days of age was proportional to the animals’ increase in body mass, with both being representative of the rapid growth rate of juvenile rats. The difference in wet mass was largely attributable to the greater fiber size (34% greater mean fiber cross-sectional area) in the muscles of the older animals.

**MyoD-positive nuclei, new myonuclear production, and myonuclear number.** The lower frequency of MyoD-positive nuclei after the 6 days of normal growth suggested a decline in satellite cell proliferation. This is consistent with previous reports of a net decrease in the mitotic activity of satellite cells between birth and adulthood (50, 62). However, the number of MyoD-
positive nuclei expressed as a percentage of the number of myonuclei (5–8%, recalculated per fiber cross section) was a large proportion of the documented available satellite cells in fast muscles of 30- to 35-day-old rats (7–9% of the myofiber nuclear population; Refs. 14, 27). This suggests either that the majority of the MyoD-positive nuclei were satellite cells and a larger than expected (62) proportion of these were mitotically active, or that some MyoD-positive nuclei were myonuclei within the growing fibers.

The fusion of a proportion of the proliferating cells with their host fiber is believed to contribute to the increase in the number of myonuclei during muscle growth (50). We found a nonsignificant 5% difference in the number of myonuclei per millimeter fiber length between the 30- and 36-day-old muscles. This difference is proportionately similar to the 10% difference between 30 and 40 days of age in rat extensor digitorum longus (EDL) fibers (62). The detection of a statistically significant difference in this variable would require the use of rats with a greater age difference, as the mean number of myonuclei per fiber length in fast muscles increases only gradually from a few weeks after birth until young adulthood (41, 61).

The accretion of newly produced myonuclei, as demonstrated by the number of BrdU-labeled nuclei within the isolated muscle fibers, was also small and unexpectedly lower than the difference in the number of myonuclei per fiber length between the 30- and 36-day-old muscles. Most newly replicated satellite cell nuclei and, therefore, newly produced myonuclei within the fibers should have been labeled with the BrdU (62) and thus nearly fully account for the difference in myonuclear number. It is possible that the differences in new myonuclear production and myonuclear number during the short experimental period were both too small to detect reliably. However, we have yet to fully explain this discrepancy. Regardless, these data and previously reported increases in myonuclear number are much smaller than the corresponding increases in muscle mass, fiber mass, or cross-sectional area during postnatal growth, particularly in the predominantly fast EDL and gastrocnemius muscles (23, 28, 41, 61). This suggests that the importance of increasing myonuclear number in enabling increases in fiber size and the maintenance of a constant myonuclear domain are less during maturational growth than that reported for adult muscle hypertrophy (1, 59).

Effects of Btx-induced Paralysis on Juvenile Gastrocnemius Muscle

We found effects of Btx on myocellular events related to growth of the muscle, yet no direct structural effects could be seen at the light microscopic level at the early time point examined, consistent with ultrastructural observations in adult mouse gastrocnemius (18). Thus we have no evidence to suggest that the observed effects of the toxin were mediated through any mechanism other than the paralysis of the muscle fibers.

Muscle wet mass and fiber size. Btx injection inhibited the age-related increase in muscle wet mass and resulted in a 32% smaller mean fiber size than in the contralateral NoBtx muscles after 7 days. This rapid effect is similar to that described previously in 3-wk-old rats, where Btx injection resulted in a 32% reduction in gastrocnemius wet mass and a 26% smaller fiber diameter, despite functional paralysis remaining for only up to 10 days (6). Similarly, 9 days of tetrodotoxin (nerve block)-induced paralysis in 3-wk-old rats resulted in a 40% decrease in gastrocnemius wet mass and prevented any age-related increase in fiber area (45). Clearly, chemoparalysis of young, fast muscle rapidly arrests muscle and fiber growth and soon leads to muscle and fiber atrophy.

MyoD-positive nuclei, new myonuclear production, and myonuclear number. The observed twofold greater frequency of MyoD-positive nuclei in Btx vs. NoBtx muscles we interpreted as due, at least in part, to an enhanced activation and proliferation of satellite cells 7 days after the injection. Increases in mitotic cell (including satellite cell) activity due to Btx injection have also been reported in the laryngeal muscles of adult rats after 3 and 7 days, with a return to baseline after 28 days (36). This early effect of Btx-induced paralysis is similar to that seen in muscles soon after denervation (47, 53) or spinal cord transection (19, 20) in adult rats.

Relative to the number of myonuclei within the fibers, the number of MyoD-positive nuclei was large compared with the expected proportion of satellite cells in the muscle. Thus, barring a substantial increase in the number of satellite cells, and a large proportion thereof being within the cell cycle, a proportion of the observed MyoD-positive nuclei would not have been satellite cell nuclei. It is unlikely that many MyoD-positive nuclei were newly fused myonuclei, because the number of BrdU-positive myonuclei in the Btx muscle fibers was low and not different from that of the NoBtx muscles. However, mature myonuclei have been reported to express MyoD under conditions such as chronic stretch or denervation (43, 66). Thus the extent of the increase in satellite cell activation and proliferation was probably less than the twofold difference seen in the number of MyoD-positive nuclei between the Btx and NoBtx muscles.

Our results suggest that juvenile fast muscle has some capacity to increase satellite cell proliferation, at least in the short term, in response to inactivation of the muscle. The mitotic response of satellite cells thus appears to not be limited to either stimulation by the products or consequences of physical disruption of the nerve or to disinhibition by the removal of neuromuscular contacts or trophic exchange. The prevention of neuromuscular transmission alone, by the injection of Btx, is sufficient for this effect.

The limited number (i.e., 1–2%) of BrdU-labeled myonuclei within fibers after 7 days suggests that, despite an increased activation and proliferation of satellite cells in the Btx muscles, the progression of these cells through the cell cycle and/or their exit to...
form new myonuclei was either prevented or delayed. This discrepancy of a higher satellite cell activity than production of new myonuclei still remains, although to a smaller extent, if the MyoD-positive nuclei observed were not exclusive to satellite cells. We also acknowledge that this comparison is between variables measured in muscle sections and in isolated fibers taken from the same muscle. Thus it is possible that regional differences in these properties might account, at least in part, for the increase in MyoD-positive nuclei coincident with a low number of newly produced myonuclei. However, this apparent inability, but for a very small proportion of the MyoD-expressing nuclei, to translate into an increase (or reduced decrease) in myonuclear number is consistent with previous observations in adult slow (soleus) muscle paralyzed by spinal cord transection (20). The removal of neuromuscular activity by Btx may thus prevent appropriate signals for cell cycle progression and fusion, such as the expression or availability of specific autocrine growth factors or cell-surface proteins.

In addition to a low production of new myonuclei, we found a 22% loss in the number of myonuclei per millimeter fiber length between the Btx and NoBtx muscles. A similar rapid and significant loss of myonuclei during early muscle atrophy is consistent with prior reports from adult slow muscles. However, several studies of fast muscle fibers have demonstrated only small or nonsignificant changes in the number of myonuclei (2, 20, 21, 40, 61). One report of reduced activity in juvenile gastrocnemius muscle has described a 16% greater number of myonuclei per millimeter fiber length, coincident with a 14% smaller fiber size, in 5-day space-flown rats, compared with age-matched, weight-bearing controls. However, muscles from 5-day hindlimb-suspended rats showed the same degree of fiber atrophy but no significant difference in myonucleus number from the controls (40). Recently, Dupont-Versteegden et al. (21) and Schmalbruch and Lewis (61) have described a maintenance of myonuclear number after 8 wk of paralysis in the adult rat plantaris and after up to 22 wk of denervation in the EDL of young rats, respectively. Differences between our data and the aforementioned studies may be due to the blockage of most of the neurally mediated activity by the Btx and/or differences in the chosen experimental techniques. Our data using Btx support an essential role for electrical transmission between the nerve and the muscle fiber in controlling changes in myonuclear production and number during postnatal growth.

To the best of our knowledge, the present data are the first to describe a loss of myonuclei per unit length in fibers from juvenile fast muscle. This adds further numerical evidence of selective nuclear elimination in the absence of overt fiber necrosis in nonpathological, atrophic muscle. Furthermore, the myonuclear number and fiber size of the Btx muscles were both smaller than those in the NoBtx muscles to a similar extent. This is in contrast to a number of models of adult muscle inactivity that have shown fiber atrophy in excess of myonuclear loss (1, 2, 64). However, as noted above, regional differences within the muscle may have affected the relative magnitude of the changes measured in each of these variables.

**Effects of Exercise on Juvenile Gastrocnemius Muscle**

The nature of the exercise is important to the cellular and molecular adaptations in the muscle and presumably the interactions with processes of normal postnatal muscle growth. The daily running distances that we observed were low (8), although the large interanimal variability was to be expected (49, 57). However, spontaneous wheel-running activity and treadmill running at similarly fast speeds (e.g., 30–60 m/min) are of sufficient intensity to recruit fast fibers of the ankle extensor muscles (5, 57). The present protocol also included the mass of the pump on the rats’ dorsum that would have provided an additional weight-bearing load. At insertion, this was 7% of the rats’ body mass.

**Muscle wet mass and fiber size.** The exercise had a significant growth-enhancing effect on the young muscle wet mass (18%) and mean fiber size (23%). This effect has also been noted in the juvenile plantaris muscle, in which increases in wet mass (16%), protein content, and fractional synthesis rate after 2 wk (52) and increases in fast fiber cross-sectional area after 45 days (38) of voluntary wheel running were found.

**MyoD-positive nuclei, new myonuclear production, and myonuclear number.** The wheel-running exercise had no effect on the number of MyoD-positive nuclei in either NoBtx or Btx muscles. In the NoBtx muscles, it is likely that more intense or unaccustomed exercise capable of causing minor muscle fiber injury is required to increase satellite cell proliferation, as has been shown after downhill treadmill running in the juvenile rat EDL (13). Similarly, the lack of an exercise effect on the number of BrdU-positive myonuclei was consistent with our laboratory’s previous data from the adult fast ankle flexor muscle (tibialis anterior) after 7 days of daily downhill treadmill exercise (65).

Furthermore, while enhancing fiber size, the exercise had no influence on the number of myonuclei per fiber length. This is in contrast to the general coupling of increases in myonuclear number with increases in fiber size in adult muscle, albeit after longer periods of time and with chronic and/or intense (overload/resistance-type) exercise (1, 59). Our data suggest that the fiber-size effect preceded detectable increases in myonuclear number or that the two variables are only conditionally or indirectly related such that exercise-induced increases in the myonuclear domain are possible in juvenile muscle.

**Effects of Exercise on Paralyzed Juvenile Gastrocnemius Muscle**

We believe that this is the first report to describe cell growth-related processes in juvenile muscle that has undergone concurrent paralysis and increased (passive) activity. The increased limb use attenuated the
atrophy associated with the Btx-induced paralysis but had no effect on the changes in the number of MyoD-positive nuclei, new myonuclear production, or myonuclear number 7 days after the injection of the toxin.

Importantly, the growth-enhancing effect of the exercise in the paralyzed muscle was over and above that due to normal maturation alone and similar in magnitude to that seen in the NoBtx muscles. The collective effect of the exercise on the Btx-injected muscle, including the passive movement of paralyzed fibers and directly or indirectly related events, such as the release of local growth factors, was as effective in increasing mean fiber size as the nerve-stimulated, active contraction of the fibers within the nonparalyzed, contralateral muscle.

Despite the large variation in the total distance run among the Btx-Ex rats, post hoc analyses showed no correlation between this distance and the relative magnitude of the difference between the two limbs of each rat for each variable tested (all P > 0.28).

Muscle wet mass and fiber size. Previous studies have demonstrated varied effects of exercise on the wet mass or fiber size of paralyzed muscle (17, 25, 32, 37). The method and degree of paralysis, the extent of subsequent reinervation of the muscle, and the time of onset, duration, and intensity of the exercise training used in the different studies may explain the lack of consistent findings. In the present study, the muscle paralysis was evident the day after the injection and was present throughout the remainder of the experimental period. Furthermore, because of the method by which we administered the toxin and the observed effects on fiber size, we believe that the majority of the fibers within the gastrocnemius would have been paralyzed. The exercise was, therefore, concurrent with near or complete paralysis of the muscle.

The prolonged effects of exercise on fully paralyzed muscle have been equivocal. Six weeks of treadmill running (0.5–1.4 km/day) had no effect on either type I (slow) or IIa (fast) fiber size in the permanently denervated soleus muscle (17). However, daily cyclic stretch of permanently denervated rat EDL muscle for 23 days reduced, by about one-fourth, the severity of the atrophy in type II fibers (55). Daily passive cycling exercise of paralyzed limbs for the final 4 of 8 wk after thoracic spinal cord transection in adult rats had no significant effect on mean fiber areas in the soleus muscle but attenuated the fiber atrophy in the fast plantaris (21).

The short-term effects of exercise on paralyzed, adult rat muscle have also been documented (19). Significant muscle atrophy was found 10 days after spinal cord transection, whereas 60 min of daily passive exercise, for the last 5 of the 10 days, attenuated the atrophy of type IIa and IIId/x fibers in the EDL and type I and IIa fibers in the soleus (19). Our data are thus consistent with evidence that passive exercise of paralyzed muscle can have growth-promoting (or atrophy-inhibiting) properties and demonstrate this effect 7 days after Btx injection in juvenile fast muscle. Thus, in the absence of neuromuscular transmission and active contraction of the extrafusal and intrafusal muscle fibers, there are sufficient factors and events stimulated directly or indirectly by the muscle activity during the wheel running to attenuate fiber atrophy.

MyoD-positive nuclei, new myonuclear production, and myonuclear number. Despite attenuating the atrophy, increased limb use was not associated with changes in the number of MyoD-positive nuclei or the production of new myonuclei nor did it prevent the myonuclear loss in the paralyzed muscle. Passive exercise after spinal cord transection (as previously described) also failed to affect the level of MyoD mRNA or the accumulation of MyoD in satellite cell nuclei in the adult EDL or soleus muscles (19). Further studies in the more responsive soleus confirmed that the exercise had no effect on the increase in satellite cell proliferation, as determined by the incidence of myogenin-positive nuclei, in the paralyzed muscle (20). These observations suggest that the movement or tension on the muscle during the exercise had either a stimulatory or counterinhibitory effect on alternative fiber growth-related processes, such as protein accretion. It is well known that stretch of fast muscle stimulates protein synthesis and fiber hypertrophy, independent of nerve-evoked activity (29).

Collectively, our results demonstrate that Btx injection-induced paralysis leads to increases in the number of MyoD-positive nuclei, which is suggestive of an enhanced satellite cell proliferation, myonuclear loss, and fiber atrophy in juvenile gastrocnemius muscle. Exercise of the paralyzed muscle cannot prevent these changes but has either stimulatory or counterinhibitory effects, independent of normal muscle contractile activity, on other fiber growth-related processes. Similarly, the increased activity of nonparalyzed muscle did not appear to influence the number of MyoD-positive nuclei or the production and accretion of myonuclei per fiber length yet did enhance net growth within the fiber. These results using Btx suggest that electrical transmission at the neuromuscular junction is important to the maintenance or increase in myonuclear number, whereas increased neurally mediated contractile activity by voluntary exercise does not result in an enhancement of myonuclear accretion, during fiber growth. The ability to either inhibit the loss or increase the production of myonuclei may not be of direct or immediate importance to the regulation of fiber size in fast muscle early following the onset of paralysis and concurrent daily exercise. Such changes might instead be considered potential longer term determinants of fiber growth and adaptation (13, 51). We are now examining a more extensive time course of the events contributing to muscle fiber growth in Btx-injected and/or exercised muscle.

In conclusion, Btx-induced paralysis compromised muscle growth and led to fiber atrophy 7 days postinjection. Exercise enhanced normal age-related increases and attenuated the Btx-induced losses in muscle and fiber size. The changes in fiber size due to the Btx and/or exercise were greater in magnitude and neither highly nor consistently associated with alterations in the incidence of MyoD-positive nuclei, new
myonuclear production, or myonuclear number per fiber length, in the gastrocnemius muscle of juvenile rats.

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REFERENCES


