Ontogenetic, gravity-dependent development of rat soleus muscle

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Department of Physiology and Biomechanics, Research Center for Sports Training and Education, National Institute of Fitness and Sports, Kanoya, Kagoshima 891-2393; Department of Laboratory Medicine, National Center for Neurology and Psychiatry, Kodaira, Tokyo 187-8551, Japan; and Department of Physiological Science and Brain Research Institute, University of California, Los Angeles, California 90095

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Ohira, Yoshinobu, Takato Tanaka, Tomoo Yoshinaga, Fuminori Kawano, Takeshi Nomura, Ikuya Nonaka, David L. Allen, Roland R. Roy, and V. Reggie Edgerton. Ontogenetic, gravity-dependent development of rat soleus muscle. Am J Physiol Cell Physiol 280:C1008–C1016, 2001.—We tested the hypothesis that rat soleus muscle fiber growth and changes in myosin phenotype during the postnatal, preweaning period would be largely independent of weight bearing. The hindlimbs of one group of pups were unloaded intermittently from postnatal day 4 to day 21: the pups were isolated from the dam for 5 h during unloading and returned for nursing for 1 h. Control pups were either maintained with the dam as normal or put on an alternating feeding schedule as described above. The enlargement of mass (~3 times), increase in myonuclear number (~1.6 times) and myonuclear domain (~2.6 times), and transformation toward a slow fiber phenotype (from 56 to 70% fibers expressing type I myosin heavy chain) observed in controls were inhibited by hindlimb unloading. These properties were normalized to control levels or higher within 1 mo of reambulation beginning immediately after the unloading period. Therefore, chronic unloading essentially stopped the ontogenetic developmental processes of 1) net increase in DNA available for transcription, 2) increase in amount of cytoplasm sustained by that DNA pool, and 3) normal transition of myosin isoforms that occur in some fibers from birth to weaning. It is concluded that normal ontogenetic development of a postural muscle is highly dependent on the gravitational environment even during the early postnatal period, when full weight-bearing activity is not routine.

muscle fiber development; myosin phenotype; myonuclei; gravitational loading

THE MANIFESTATIONS OF GRAVITATIONAL INFLUENCES on the basic contrasting designs of neuromotor systems among species are abundant. Numerous asymmetries in the neural organization and the muscles of appendages that are responsible for locomotion and posture on Earth in aquatic, amphibian, and terrestrial animals have been described (18). Chronic unloading of the hindlimbs of rats during the postweaning period when full weight bearing is routine results in severe and rapid atrophy of the soleus muscle (17). One might assume that the evolution of these gravity-oriented asymmetries would be programmed largely independent of the level of mechanical loading, at least during the early neonatal period. However, the extent that the Earth’s 1G gravitational environment defines those properties ontogenetically to accommodate terrestrial locomotion during the early postnatal period of development is unknown (9, 29–32). We asked the question, Have those gravitational influences that have been shaped phylogenetically become ontogenetically independent of gravity during the immediate postnatal developmental period?

In the process of normal development during the neonatal period of rats, i.e., from birth (day 0) to weaning (day 21), several major cellular-intercellular events proceed in muscles that eventually define their functional properties at the weaning age of ~21 days. Muscle (and muscle fiber) growth and changes in myosin phenotype clearly are responsive to the absence of load bearing during the postweanening period (7, 10, 15, 23, 24). We hypothesized that the normal development of muscle mass, fiber size, and myosin phenotype would be independent of gravitationally imposed loading during the preweaning period. We also hypothesized that the mitotic events leading to an increase in the number of myonuclei would occur independently of load bearing during development. This seemed reasonable because full weight bearing of the hindlimbs does not occur routinely until ~15 days of age in the rat (8).

METHODS

Experimental Design and Animal Care

All experimental procedures were conducted in accordance with the Japanese Physiological Society Guide for the Care
and Use of Laboratory Animals, approved by the Committee on Animal Care and Use at the National Institute of Fitness and Sports, and followed the guiding principles of the American Physiological Society. Adult male and female Wistar rats were mated and fed a commercial solid diet (CE-2, NihonCLEA, Tokyo, Japan) and water ad libitum. The pups (~13/litter) in each litter were separated randomly into five groups: 1) vivarium control; 2) isolated control (same schedule as group 4); 3) isolated control and reambulation (same schedule as group 5); 4) hindlimb unloaded by tail suspension beginning on postnatal day 4 and unloaded until day 21; and 5) hindlimb unloaded as in group 4 and then allowed to reambulate for 28 days. Water and both solid and powdered diets were supplied during the last week of the nursing period. All animals were housed in cages with wood shavings at all times. In addition, all pups were handled with rubber gloves at all times to avoid rejection by the dam.

For all hindlimb-unloaded pups, a narrow piece of tape was secured to the lower third of the tail and remained so throughout the duration of the unloading period. A second piece of tape was attached to the string, i.e., the piece fixed to the string, was removed. Pups in the isolated control group also were separated from their mother and followed a similar feeding schedule. Pups in the vivarium control group were kept with their mothers throughout the nursing period. During the reambulation period of groups 3 and 5, the rats were separated from their mothers and were pair-fed a solid diet. The amount of food supplied for each rat, which was completely eaten within ~12 h, was gradually increased from ~6 to ~20 g in accordance with growth. Temperature and humidity in the animal room with a 12:12-h light-dark cycle were maintained at ~23°C and ~55%, respectively. Hindlimb-unloaded and isolated control rats were killed at either postnatal day 9 or 21 (after 5 or 17 days of unloading) or 49 (after 17 days of unloading plus 28 days of reambulation) (n = 6/day). Vivarium control rats were killed at either 4, 9, 21, or 49 days of age (n = 6/day). Because body and muscle weight and fiber size and phenotype distribution were similar in the isolated and vivarium control groups (see Table 1), the control group in Figs. 1–7 and in the text refers to the isolated control group unless vivarium control is specifically stated.

**Muscle Preparation**

Rats were anesthetized intraperitoneally with pentobarbital sodium, and the soleus muscles were removed bilaterally. The hindlimb-unloaded rats were anesthetized while the hindlimbs remained unloaded to avoid any effects of acute loading. The muscles were cleaned of excess fat and connective tissue and were wet weighed immediately. For immunohistochemical analyses, the muscles were pinned on a cork at approximately resting length and frozen in isopentane cooled with liquid nitrogen. Each muscle was mounted on a cork by approximately 10–12 h dark-light cycle and freezing in ~100°C.

**Analysis of Myonuclear Number**

Single muscle fiber segments were prepared as described by Allen et al. (4, 5, 7). An average of 44 fibers/muscle was stained for 5 min with 54 μM acridine orange and then for 5 min with 1.5 × 10−7 M propidium iodide in phosphate-buffered saline. The combination of these fluorescent dyes produces the best staining and contrast between the cytoplasm (acridine orange) and the myonuclei (propidium iodide). A Sarastro 2000 Laser Scanning Confocal Microscope (Molecular Dynamics, Sunnyvale, CA) was used to analyze fiber cross-sectional area (CSA), myonuclear number, and cytoplasmic volume per myonucleus (myonuclear domain) (11, 19).

First, a series of scans was taken through the entire Z thickness of a fiber region with the proper filter sets for acridine orange fluorescence. Myonuclear number was determined by counting all the myonuclei in the stack, with the length of the field being ~173 μm, and converting to myonuclei per millimeter. Next, a maximum-intensity projection rotated orthogonally to the long axis of the fiber was produced from the stack, and fiber CSA was calculated as described previously (7).

To correct for possible effects of different states of fiber stretch, 10 consecutive sarcomere lengths of the fiber were measured in three nonoverlapping regions and averaged. Both myonuclei per millimeter and CSA were normalized to a 2.5-μm sarcomere length. Fiber ends, damaged regions, and excessively stretched (sarcomere lengths >3.5 μm) regions were omitted from the analyses.

**Single-Fiber Gel Electrophoresis**

After confocal analysis, gel electrophoresis was carried out in the same single-fiber segments, as previously described (4, 5, 7). To facilitate an accurate determination of MHC isoform expression, two lanes were loaded with 10 μl of a mixture of homogenate of medial gastrocnemius and soleus muscles of adult rats. The gels were run overnight at 80 V in an ice-packed cooler. Gels were stained with Rapid Coomassie (Diversified Biotech, Boston, MA).

**Immunohistochemical Analyses of Muscle Fiber Types and Size**

The expression of MHCs in individual fibers was analyzed in serial cross sections by using monoclonal antibodies specific to fast or slow MHC isoforms, i.e., primary antibody, NCL-MHCs and NCL-MHCF (Novocastra Laboratories, UK), as described previously (26). The avidin-biotin immunohistochemical procedure was used for the localization of primary antibody binding according to the instructions for kits PK-6102 and AK-5010 (Vector Laboratories, Burlingame, CA). Phosphate-buffered saline was used as a buffer for all immunoglobulin G-class primary antibodies, and tris(hydroxy-methyl)aminomethane-buffered saline was used as the buffer for all immunoglobulin M-class primary antibodies. The stained images were incorporated into an image processing system (Color Image Processor, SPICCA-II; Nihon Avionics, Tokyo, Japan). Approximately 120 fibers were analyzed in each muscle sample. The fiber types were classified as type I, II, or I+II (hybrid) according to the staining profile (26). In addition, the CSA of each fiber was determined.

In addition, whole soleus muscles of all 4-, 9-, and 21-day-old control and unloaded rat pups were homogenized in buffer solution composed of 250 mM sucrose, 100 mM KCl, 5 mM EDTA, and 20 mM Tris (pH 6.8). The MHC expression in these homogenates was analyzed by using gel electrophoresis...
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I
II, and II MHC fibers in the vivarium and isolated
in Fig. 1. For example, the mean percentage of type I,
trols, only data from the isolated control are included
butions were similar in the vivarium and isolated con-
Immunohistochemical Properties of Muscle Fibers
were similar in the three groups at 49 days of age.

Body and Muscle Weights
The body and soleus weights of the vivarium control,
isolated control, and hindlimb-unloaded groups of rats
did not change significantly between days 4 and 9 after
birth (Table 1). The mean body weights of these groups
were significantly increased approximately twofold
from day 4 to day 21, and there was no significant
differences among the three groups. Whereas the mean
soleus weight of both groups of control rats increased
approximately fourfold between days 4 and 21, the
soleus weight in the hindlimb-unloaded rats was un-
changed. After 1 mo of reambulation, however, the
mean muscle weight of the previously hindlimb-un-
loaded rats was increased to that of the age-matched
control rats. Both the mean body and muscle weights
were similar in the three groups at 49 days of age.

Immunohistochemical Properties of Muscle Fibers
Fiber phenotype. Because the fiber phenotype distribu-
tions were similar in the vivarium and isolated con-
trols, only data from the isolated control are included
in Fig. 1. For example, the mean percentage of type I,
I+II, and II MHC fibers in the vivarium and isolated
controls at 21 days of age were 71.2 ± 4.4 vs. 70.4 ±
5.1%, 1.9 ± 0.2 vs. 0.9 ± 0.1%, and 26.8 ± 2.3 vs. 28.4 ±
2.3%, respectively. In the control rats, the percentage
of fibers expressing pure type I MHC tended to in-
crease (P = 0.08), and that of fibers containing only
type II MHC decreased, during the nursing period. In
contrast, the distribution of MHC fiber phenotypes was
unchanged in response to unloading. There was a rel-
atively small percentage of hybrid fibers in both groups
at all time points.

Fiber size. The mean fiber CSA was similar in the
vivarium and isolated controls. For example, the mean
CSA in type I, I+II, and II MHC fibers in the vivarium

Table 1. Body weight and soleus muscle weight

<table>
<thead>
<tr>
<th>Age, days</th>
<th>VC</th>
<th>VC</th>
<th>IC</th>
<th>Unloaded</th>
<th>VC</th>
<th>IC</th>
<th>Unloaded</th>
<th>VC</th>
<th>IC</th>
<th>Unloaded</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW, mg</td>
<td>18.0 ± 0.5</td>
<td>18.7 ± 0.4</td>
<td>20.3 ± 0.2</td>
<td>19.0 ± 1.2</td>
<td>41.6 ± 1.8*</td>
<td>36.0 ± 1.3*</td>
<td>33.5 ± 1.6*</td>
<td>33.2 ± 1.6*</td>
<td>33.2 ± 1.6*</td>
<td>36.4 ± 1.1**</td>
</tr>
<tr>
<td>MW, mg</td>
<td>2.8 ± 0.2</td>
<td>2.8 ± 0.2</td>
<td>3.1 ± 0.1</td>
<td>2.9 ± 0.1</td>
<td>12.3 ± 0.9*</td>
<td>12.4 ± 0.5*</td>
<td>3.7 ± 0.4</td>
<td>3.7 ± 0.4</td>
<td>3.7 ± 0.4</td>
<td>3.7 ± 0.4</td>
</tr>
<tr>
<td>%BW*10^-3</td>
<td>15.6 ± 0.1</td>
<td>18.1 ± 0.5</td>
<td>15.2 ± 0.5</td>
<td>15.2 ± 0.1</td>
<td>30.0 ± 1.1*</td>
<td>34.2 ± 0.9*</td>
<td>12.1 ± 1.1</td>
<td>39.2 ± 1.2**</td>
<td>39.2 ± 1.2**</td>
<td>42.3 ± 2.0**</td>
</tr>
</tbody>
</table>

Values are means ± SE, n = 6 rats in each group. BW, body weight; MW, muscle weight; VC, vivarium control; and IC, isolated control. The 49-day-old hindlimb-unloaded group was hindlimb unloaded from day 4 to day 21 and reambulated for 28 days. *P < 0.05 vs. 4-day-old VC rats. †P < 0.05 vs. 9-day-old IC rats. §§P < 0.05 vs. 21-day-old IC rats. §§§P < 0.05 vs. age-matched IC rats.

as described above. A mixture of homogenate of medial gas-
trocnemius and soleus muscles of both adult and neonatal
rats (9 days old) was used for the standard.

Statistical Analysis
Values are expressed as means ± SE. Significant differ-
ences between groups were determined by using analysis
of variance followed by Scheffe’s post hoc test. Standard regres-
sion and correlation procedures were used to determine the
relationships between CSA and myonuclear number or myo-
nuclear domain. Statistical significance was set at P < 0.05.

RESULTS

Body and Muscle Weights

Fiber size. Soleus muscle fiber size increased during the 3-wk nursing period in control rats (Fig. 3A). How-
ever, no increase in fiber size occurred in the fibers of
hindlimb-unloaded rats during the same period. In
control rats, the overall (all MHC phenotypes com-
bined) mean fiber size was unchanged between postna-
tal days 4 and 9. The mean CSA of each fiber type significantly increased approximately three- to fourfold between days 9 and 21 in control. In contrast, there was no increase in the
CSA of any fiber type in the hindlimb-unloaded rats
during this same time period.

Confocal Microscopic and Electrophoretic Analyses:
Non-Phenotype-Specific Responses

Fig. 1. Mean percentage of soleus muscle fibers expressing type I, I+II, or II myosin heavy chain (MHC) isoforms (classified by a fast
and a slow MHC monoclonal antibody) in isolated control and hind-
limb-unloaded rats at 4, 9, and 21 days of age. Rats were hindlimb
unloaded at 4 days of age (thus there is no hindlimb-unloaded group
at this age). Values are means ± SE; n = 6 rats in each group. The
mean number of fibers analyzed for each rat was 120. *P < 0.05 vs.
4-day-old rats. §§P < 0.05 vs. age-matched control rats.
loaded rats was similar to that observed in 4-day old control rats, the time at which the unloading treatment was initiated. However, within 4 wk of reambulation, the mean fiber size tended to be larger ($P < 0.14$) than in the age-matched controls.

Myonuclear number. The number of myonuclei in soleus fibers of control, but not hindlimb-unloaded, rats increased during the 3-wk nursing period (Fig. 3B). Myonuclear number per millimeter for all MHC phenotypes combined in control rats was similar at postnatal days 4 and 9 but was significantly increased by $\sim 50\%$ from day 9 to day 21. In contrast, the number of myonuclei per millimeter in 9- and 21-day hindlimb-unloaded rats was similar to that observed in 4-day-old control rats. The myonuclear number in the unloaded group tended to be higher than that in the age-matched control levels after 1 mo of reambulation ($P = 0.55$).

Myonuclear domain. The overall mean myonuclear domain for all fibers in control rats was similar at postnatal days 4 and 9 and significantly increased by $\sim 130\%$ from day 9 to day 21 (Fig. 3C). In contrast, the mean overall cytoplasmic volume per myonucleus in 9- and 21-day hindlimb-unloaded rats was similar to that observed in 4-day control rats. After 1 mo of reambulation, the myonuclear domains were significantly increased, and the values were greater than control values.

Confocal Microscopic and Electrophoretic Analyses: Phenotype-Specific Responses

Fiber phenotype. At 21 days of age, there was a slightly lower ($P = 0.11$) percentage of pure type I MHC fibers (54 vs. 70%) and a higher percentage of fibers that contained more than two MHC isoforms in hindlimb-unloaded rats than in control rats (Fig. 4). A major difference was that 16% more fibers contained some type IIx MHC isoform in the hindlimb-unloaded rats compared with the control rats, with the percent-age of fibers expressing type I+IIa+IIx MHC being significantly higher in hindlimb-unloaded rats than in control rats. In addition, a de novo appearance of fibers coexpressing all four MHC isoforms (I, IIa, IIx, and IIb) was observed in the soleus of the hindlimb-unloaded rats.

The unloading-related effects on fiber phenotype were normalized after 1 mo of ambulation recovery. At this stage, only three types of fibers were observed in both groups, i.e., fibers containing only type I MHC, only type IIa MHC, or both type I and IIa MHC. Furthermore, the percentage of each type was similar in the two groups.
Fiber size. The mean fiber CSA in the 21-day-old hindlimb-unloaded rats, regardless of MHC profile, was \( \sim 250 \, \mu m^2 \), i.e., about one-half the size of the fibers in 21-day-old control rats (Fig. 5A). The CSAs of muscle fibers containing type I, I+IIa+IIx, or IIa+IIx MHC were significantly smaller in hindlimb-unloaded rats than in control rats. Muscle fiber growth recovered to the age-matched control levels during the ambulation period. Interestingly, the mean CSA of pure type I and hybrid (I+IIa MHC) fibers was 33% and 15% \( (P = 0.12) \) larger, respectively, in reambulated rats than in age-matched control rats.

Myonuclear number. The fiber type-specific responses in myonuclear number per millimeter are shown in Fig. 5B. There were fewer myonuclei per millimeter in 21-day-old hindlimb-unloaded fibers than in age-matched control fibers, with the difference being significant for pure type I and type IIa+IIx MHC fibers. After 1 mo of reambulation, the number of myonuclei per millimeter recovered to the age-matched control levels. In fact, the number of myonuclei was significantly higher in pure type I MHC fibers of previously hindlimb-unloaded rats than in those of age-matched control rats.

Myonuclear domain. The trends for changes in myonuclear domain after 17 days of unloading during the nursing period were similar to those for fiber size and myonuclear number across all fiber types (Fig. 5C). The mean myonuclear domains tended to be smaller in the hindlimb-unloaded rats than in the control rats, but the difference (\( \sim 40\% \)) was significant only for fibers coexpressing I+IIa+IIx MHC isoforms. A significant “supercompensation” effect after 1 mo of recovery was evident in the myonuclear domain size of the pure type I MHC fibers, consistent with a similar significant effect on fiber CSA (Fig. 5A) and myonuclear number (Fig. 5B). In addition, the mean myonuclear domains of pure type IIa or I+IIa MHC fibers in previously hindlimb-unloaded rats were significantly larger than those in age-matched control rats.

Fig. 4. Mean percentage of fiber types (classified by single-fiber gel electrophoresis) of the soleus muscle of 21- (21C) and 49-day-old isolated control rats (49CR) and 21- (21U) and 49-day-old hindlimb-unloaded rats (49UR). Unloaded rats had their hindlimbs unloaded for 17 days, i.e., from 4 to 21 days of age. A group of hindlimb-unloaded rats and a group of control rats then were allowed to reambulate for 28 days. Values are means ± SE; \( n = 6 \) rats in each group. The mean number of fibers analyzed for each muscle was 44. *\( P < 0.05 \) vs. 21-day-old control rats.

Fig. 5. Mean CSA (A), myonuclear number (B), and myonuclear domain (C) of each fiber type in the soleus muscle. The groups, fiber types, and numbers of rats and muscle fibers analyzed for each rat are the same as in Fig. 4. Values are means ± SE; \( n = 6 \) rats in each group. *\( P < 0.05 \) vs. 21-day-old control rats. †\( P < 0.05 \) vs. 21-day-old hindlimb-unloaded rats. §\( P < 0.05 \) vs. 49-day-old (age matched) control rats.
Relationship between fiber size and myonuclear number or domain. Close relationships between fiber CSA and myonuclear number (Fig. 6) and fiber CSA and myonuclear domain (Fig. 7) were observed at the end of the nursing and unloading periods. Positive correlations between these parameters were maintained even during the period of "supercompensatory" fiber growth associated with reambulation.

DISCUSSION

The soleus muscle fibers failed to grow in the absence of loading during a period when fiber size would normally increase more than threefold. The failure of the soleus muscle fibers to grow in the unloaded pups was not related to malnutrition effects caused by intermittent feeding, as was reported elsewhere (20, 28). There was no statistically significant difference in the mean body or soleus weights of the pups allowed free access to the dams (vivarium controls) and those pups that had the same interrupted access to the dams (isolated controls) as the hindlimb-unloaded pups. In addition, the mean fiber sizes and phenotype distributions were similar in these two control groups.

Neither body weight nor soleus muscle weight of control pups increased between 4 and 9 days after birth, as was reported elsewhere (13). During this period of development the hindlimbs of the pups move but have minimal weight-supporting function (8). Rat pups begin to walk, jump, and run at \(11, 12,\) and 14 days after birth, respectively (12). One of the major causes of the unloading-related inhibition of growth of soleus muscle may be due to the fact that the ankle joints are plantarflexed during hindlimb unloading. Thus tension development of soleus may be inhibited because of the passively shortened muscle length (22).

In control rats the percentage of muscle fibers expressing only the slow type I MHC isoform tended to increase, and that of fibers expressing some fast type II MHC isoforms decreased, during the first 3 wk of life. At 21 days of age, \(16\%\) fewer pure type I fibers were observed in chronically hindlimb-unloaded rats than in control rats, i.e., \(54\%\) vs. \(70\%).\) Thus chronic unloading of the soleus stopped the development of muscle fiber size and reduced the percentage of fibers that would normally develop into a pure slow phenotype by \(13\%\) (from \(57\%\) to \(70\%\)) of what would have occurred with normal weight bearing. Adams et al. (1) reported an even greater effect of gravitational unloading by actual spaceflight on the distribution of type I fibers of the soleus muscle of neonatal rats. The mean percentages of type I MHC fibers in rats exposed to microgravity from age 7 to 23 days and in the age-matched controls were \(34\%\) and \(76\%\), respectively.

Compared with control values, the number of myonuclei per muscle fiber can increase as much as three times or decrease by \(>30\%\) following hypertrophy or atrophy, respectively (5, 14). The present results demonstrate that the normal development of neonatal muscle mass is indeed dependent on gravitational loading. The data also suggest that this dependence on loading is manifested as a result of an absence of the proliferation of myonuclei. One alternative explanation is that the proliferation rate remained normal, but cell death prevented the net number of myonuclei from increasing. A second possibility is that cell division was nor-
mal, but these cells could not fuse with the muscle fibers to become myonuclei. Any one of these possible interpretations leads to the same conclusion, however, and that is that the regulation of the number of myonuclei ontologically is gravity dependent.

The extent to which the modulation of the number of myonuclei in adult mammals defines the changes that occur in muscle fiber size and phenotype has been recognized only within the last few years (5, 6, 16, 25). For example, the number of myonuclei and the mean myonuclear domain in type I fibers of adult rat soleus were reduced significantly after 14 days of spaceflight (7). It seems, therefore, that a primary cellular strategy to control muscle protein metabolism and cytoplasmic volume in the adult involves not only a relatively immediate modulation via transcription of mRNAs but also a more “long-term” strategy by modulating the number of myonuclei that are available for transcription. There is some evidence that the adaptive dynamics of multinucleated muscle fibers in juvenile rats includes the activation of satellite cells as a part of the “hypertrophic” response (21, 27) and the induction of apoptosis of myonuclei in response to atrophy (3). When the hindlimbs of rats were suspended for 28 days beginning at 28 days of age (early postweaning period) (21), soleus growth was blunted but not stopped, as observed in the present study. However, in the juvenile rats there was no supercompensatory response in myofiber size, myonuclei number, or myonuclear domain after 2 or 9 wk of cage ambulation in the 28-day-old rats.

In the present study, muscle fiber size, myonuclear number, and myonuclear domain in control rats increased between postnatal days 9 and 21. In contrast, these parameters in 9- and 21-day-old hindlimb-unloaded rats remained similar to those observed in 4-day-old control rats, the age at which the unloading treatment was initiated. These results suggest that gravitational loading could have played a role in determining the mitotic potential of those cells that are thought to provide “myonuclei,” i.e., satellite and/or perhaps other stem cells, during the neonatal period of development of this slow hindlimb skeletal muscle. Furthermore, these data show that the volume of cytoplasm per myonucleus is load sensitive, even during early development, i.e., preweaning.

Do the cellular strategies for controlling fiber growth differ among fibers expressing different MHC isoforms? We were able to determine the MHC isoform profiles of isolated single-fiber segments by using gel electrophoresis procedures in rats that were 21 days of age or older. Thus in these rats we could make a clear determination of the phenotype as well as the size and myonuclear number of individual fibers. The trends for changes in fiber size, myonuclear number, and myonuclear domain were similar across fiber types. These data indicate that the effect of reduced weight bearing in neonatal rats was not unique to any specific phenotype.

Interpretation of the present data with respect to the effects of unloading during development cannot be in-
terpreted clearly with respect to the presence of neonatal and embryonic myosin isoforms. For example, we observed in whole muscle gel electrophoresis that >40% of the myosin was embryonic or neonatal at 4 days of age, ~36% at 9 days of age, and ~18% at 21 days of age. Similar levels of developmental MHC isoforms in the soleus muscle over this age range were reported by Adams et al. (2). In the present study, however, the levels of embryonic and neonatal myosin isoforms could not be determined with certainty at the single-fiber level by using either the gel electrophoresis or the immunohistochemical analyses. Although embryonic and neonatal myosins may have played a role in the responses of individual fiber phenotypes to un-bryonic and neonatal myosins may have played a role in the present data.

To determine whether the muscle properties could recover by normal postweaning weight bearing after unloading from day 4 to day 21, a group of rats was allowed to ambulate from postnatal day 21 to day 49. After 4 wk of reambulation, the soleus muscle comprised 75% pure type I, 1% pure type IIa, and 24% hybrid (containing type I and IIa MHC) fibers. No fibers with type IIx or IIb were observed. This fiber-type composition was similar to that in the age-matched controls. In addition, a supercompensation effect was evident in fiber CSA, myonuclear number, and myonuclear domain in the muscles of the reambulated rats. Thus a close relationship between fiber size and myonuclear number and domain was maintained even during the period of supercompensatory fiber growth. Combined, the present data reflect a high level of dependence of normal development of the soleus muscle on Earth’s gravitational environment. This dependence is manifested in a cellular strategy that alters the number of myonuclei available for transcription of cytoplasmic proteins to sustain fiber growth when there is normal weight-bearing function of the soleus muscle during the early postnatal period.

To examine the robustness of this cellular strategy to modulate fiber size, we compared the consistency of the relationship between fiber size and myonuclear number across several experimental conditions and with respect to MHC phenotype. While fiber sizes varied by >10-fold across these different conditions, the relationship between fiber size and myonuclear number remained similar at 21 and 49 days of age. The same relationship persisted regardless of the loading patterns imposed and, generally, regardless of MHC phenotype. These data further suggest that controlling the number of myonuclei represents a fundamental cellular strategy used under a variety of conditions to regulate muscle fiber size (6). In addition to enhancing myonuclear number, however, increasing the cytoplasmic volume per myonucleus is another important means of accommodating fiber growth. Both of these mechanisms, i.e., nuclei proliferation and cytoplasmic domain enlargement, contributed to the supercompensatory phenomenon during the postweaning reloading period. Thus, while withdrawal of normal loading at an early stage of development suppressed both mechanisms of fiber growth during the neonatal period, subsequent normal loading heightened the effectiveness of both mechanisms.

The present results suggest that during the non-weight-bearing stage of development, i.e., up to 15 days of age, Earth’s gravitational environment imposes conditions whereby some force-generating events occur during normal behavior, e.g., in competing for positioning the body to access the teats of the dam and for warmth. Perhaps the frequency of movement of the limbs when a significant level of force is generated increases gradually as the rat develops, thus preparing for the time when full weight bearing begins at ~15 days of age. Whatever forces these behaviors might presumably generate during this period, 1 of every 6 h of these behaviors was not sufficient to stimulate any significant level of muscle development or those fundamental processes that determine myonuclear number.

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