Tension- and Afferent-input-associated Responses of Neuromuscular System of Rats to
Hindlimb Unloading and/or Tenotomy

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Responses of electromyogram (EMG) in soleus muscle and both afferent and efferent neurograms at the L₅ segmental level of spinal cord were investigated during acute and chronic unloading induced by hindlimb suspension and/or tenotomy in adult rats. The soleus EMG and afferent neurogram decreased 88% and 37%, respectively, relative to those at quadrupedal posture on the floor, following acute hindlimb suspension that causes passive shortening of soleus due to ankle plantarflexion. However, the afferent neurogram (p<0.05) and soleus EMG (p>0.05) recorded on the floor increased following tenotomy of synergists. Further, the afferent input was inhibited, when the soleus EMG disappeared after tenotomy of soleus. The afferent neurogram and EMG of the soleus showed correlated responses to a variety of treatments, suggesting that the afferent neurogram recorded at the L₅ segmental level reflects the neural input associated with the activity level of the soleus predominantly. The level of efferent neurogram decreased following acute hindlimb suspension, but was not influenced significantly by tenotomy of synergists and/or soleus itself. The EMG and afferent neurograms remained low up to the 4th day, but recovered to the pre-experimental levels within 14 days, due to reorganization of sarcomere number and length, as well as the shortening of muscle fiber length and recovery of tension development. It is suggested that the levels of EMG and afferent neurogram associated with antigravity muscle are closely related to the tension development of the muscle.

Key words: soleus muscle, afferent and efferent neurogram, unloading, tension development, sarcomere remodeling
INTRODUCTION

Slow-twitch antigravity soleus muscle atrophies during exposure to microgravity (µ-g) environment (5, 22) or its simulation models such as hindlimb suspension in rats (19, 22, 24, 25). The electromyogram (EMG) of soleus disappears immediately in response to unloading by hindlimb suspension of rats (1, 19, 20, 24, 26, 28) and by exposure to actual µ-g environment, created by a parabolic flight of a jet airplane (12, 13). However, the EMG is increased gradually to the control level following 7-10 days of continuous hindlimb suspension (1, 20, 21, 24), although muscle atrophy is still induced (24, 34). Blewett and Elder (4), on the contrary, reported that suspension-related decrease in EMG did not recover during 28 days of continuous hindlimb suspension. Thus, it is still controversial how the muscular activity is influenced by gravitational unloading.

It is also reported that the activities of oxidative enzyme, succinate dehydrogenase, of motoneurons in the ventral horn of the spinal cord, which presumably innervate slow-twitch fibers, and of sensory neurons in the dorsal root ganglion were decreased following 2 weeks of spaceflight in rats (10, 11). We also reported that the afferent neurogram level recorded at the L₅ segmental level of the spinal cord decreased, when the rats were exposed to actual µ-g environment during a parabolic flight (12). These phenomena suggest that muscular adaptation to µ-g environment is closely associated with the response of nervous system. However, it is still unclear how the levels of afferent and efferent neurograms recorded at the L₅ segmental level of spinal cord are associated with the loading or unloading of soleus muscle, because the neurogram recorded at the L₅ segmental level of the spinal cord does not necessarily reflect the afferent input from the soleus muscle only (16, 27). The EMG levels of an ankle plantarflexor lateral gastrocnemius (LG) did not change significantly during a parabolic flight (12). In addition, the EMG activity of a dorsiflexor tibialis anterior (TA) tended to be even increased (p>0.05) when the rat was exposed to 20-second of µ-g. Therefore, the distribution of motoneurons innervating hindlimb muscles was investigated in
the present study.

Baker and Hall-Craggs (2) reported that tenotomy of the proximal and distal tendons of rat soleus caused shortening of muscle bellies and sarcomere length. However, the sarcomere length became comparable with that of control muscle after 4 weeks. Reduction of sarcomere length after division of the proximal tendon of mouse soleus was also reported elsewhere (15). It was, however, normalized 7 days later. The authors suggested that afferent nervous pathways are involved in the short-term adjustment of sarcomere length to fiber length. Further, it is not clear how the loading or unloading of muscle is associated the afferent and/or efferent neural input, either. Elder and Toner (7) reported that the EMG levels in rat soleus were influenced by tenotomy compared with those before tenotomy.

Therefore, the mechanisms responsible for the acute and chronic adaptation of soleus EMG and neurogram to unloading and/or shortening of muscle fiber length were investigated in rats using hindlimb suspension model in the current study. Further, effects of functional overload on soleus by tenotomy of synergists (14) or of decreased load, but with intact neural connection, by tenotomy of soleus itself were studied. The effects of tension development and/or sarcomere length on the neuromuscular activities were also investigated.

**EXPERIMENTAL PROCEDURES**

All experimental procedures were conducted in accordance with the Japanese and American Physiological Society *Guide for the Care and Use of Laboratory Animals*. This study was also approved by the Committee on the Animal Care and Use at the University and National Space Development Agency of Japan.

*Animal care*

Male Wistar rats (Kyudo, Kumamoto, Japan) with mean body weight of ~300 g were used. The experiments were performed to investigate; 1) the localization of motoneurons,
which innervate various hindlimb muscles, at the L5 segmental level of the spinal cord, 2) the effects of loading or activity levels of various hindlimb muscles on the afferent and efferent neurograms recorded at the L5 segmental level of the spinal cord, 3) the responses of soleus EMG and afferent and efferent neurograms to a continuous 14-day hindlimb suspension, 4) the effects of acute change in ankle joint angle on the soleus muscle length, tension development, and sarcomere length in control rats, and 5) the responses of the soleus muscle fiber length, sarcomere length and number, and tension development to 14 days of hindlimb suspension. Different rats were used for each investigation. A commercial solid diet (CE-2, Nihon CLEA, Tokyo) and water were supplied ad libitum. Temperature and humidity in the animal room with 12:12 hr light:dark cycle were maintained at ~23°C and ~55%, respectively.

Experiment 1: Motoneuron labeling

To map the muscle-specific motoneurons at the L5 segmental level of the spinal cord, intramuscular injections of nuclear yellow (Sigma-Aldrich, St. Louis, MO) were performed in 15 rats. The rats were anesthetized by i.p. injection of sodium pentobarbital (5 mg/100 g body weight). In 5 rats, 30 µl of 1% nuclear yellow was injected using a micro syringe into the left extensor digitorum longus (EDL) and the right medial gastrocnemius (MG). The same tracer was injected into the left TA and the right LG of other 5 rats. The left plantaris (Pl) and the right soleus in the remaining 5 rats were also injected with nuclear yellow. Care was taken to inject the fluorescent tracer slowly and prevent leakage. One day after the injection, the rats were anesthetized with i.p. injection of sodium pentobarbital, and the spinal cord and the muscles in which the nuclear yellow was injected were removed and frozen immediately in isopentane cooled with liquid nitrogen.

Serial longitudinal sections, 10 µm thick, of the spinal cord were cut in a cryostat set at −20°C. The motoneurons innervating each hindlimb muscle were identified by a golden-yellow fluorescence of the nucleus with nuclear yellow on the untreated sections using
a fluorescent microscope. All labeled motoneurons were counted and their positions plotted.

Experiment 2: Effects of hindlimb muscle activities on the neurograms

Electrode implantation. Effects of hindlimb muscle activities on the EMG and neurograms were studied in 5 rats. Detailed descriptions of the implant procedures for EMG (12, 24) and neurogram electrodes (12) were published previously. Briefly, the rat was anesthetized with i.p. injection of sodium pentobarbital, and then a skin incision was made along the sagittal suture of the skull after shaving and cleaning with betadine. The exposed skull was dried and a head plug connector was firmly anchored to the skull using both screws and dental cement. Eleven enamel-coated constantan wires, 80 \( \mu \text{m} \) in diameter, were led subcutaneously from the connector to the back region and/or left hindlimb.

The left soleus, MG and TA were exposed keeping the blood and nerve supplies intact. Bipolar electrodes were implanted into each muscle. The wires were inserted by threading individually through a 26-gauge hypodermic needle being passed through the muscle individually. The needle was carefully withdrawn and the insulated wire was stripped (~0.5 mm). The section of the wire, with the insulation removed, was implanted into the midbelly of the muscle. Two wires were inserted in parallel with the muscle fibers (~2 mm apart). The location of the wires was checked by X-ray filming for some rats in a pilot study. Further, the location of electrode was checked at the end of experiment as stated below. Each wire was secured with a suture at its entry and exit from the muscle, so that the stripped portion of the wire in the muscle was fixed.

A set of bipolar electrodes for recording of neurogram was made using tygon tubing and enamel-coated constantan wires with 80 \( \mu \text{m} \) in diameter (12). A portion of tygon tubing with 2 mm length and 1 mm inner diameter was cut longitudinally. The wires were inserted into the tygon tubing and the insulation-removed portions (~2.5 mm) were placed on the inner wall of the tubing. The end of each wire was coiled and secured using glue, so that the
stripped portion of the wire was fixed inside the tubing.

Both ventral and dorsal roots were carefully exposed at the left L₅ segmental level of the spinal cord. The afferent and efferent fibers were separated at the posterior root ganglion. The electrode apparatus was placed around the nerve fibers, keeping the stripped two wires on the neuron longitudinally (~1 mm apart), for recording of either the afferent or efferent neurogram. The end of each wire was connected to the wire led from the head plug using solder. The connected portion was insulated using enamel. A wire for the common ground was also implanted at the lower back region. A topical antiseptic (nitrofurazone, Furacin) was applied to the incision area and tetracycline was added to the drinking water (50 mg/100 ml) on the day before surgery and for 2 days to prevent infection.

Recordings and tenotomy. After 2 days of complete recovery from the surgery, the recordings of soleus, MG and TA EMG, and afferent and efferent neurograms were performed in 5 rats at quadrupedal posture on the floor (~20 seconds) and during 20-second hindlimb unloading by tail suspension followed by reloading on the floor (~20 seconds). Since the period of μ-g, which was created within each parabola, was ~20 seconds in our parabolic flight experiment (12), 20-second was chosen for the time period of acute hindlimb suspension. These recordings were performed during the same time of the day (light period, 10 a.m. – 1 p.m.). They were repeated at least 5 times and the data were averaged. At first, the EMG and neurogram recordings were performed in rats with intact tendons of hindlimb muscles. After the recording, the rats were anesthetized with i.p. injection of sodium pentobarbital, and the distal tendons of the synergists of left soleus (MG, LG, and PL) were transected keeping the blood and nerve supplies intact. On the next day, the EMG and neurogram were recorded as explained above. The rats were then anesthetized with sodium pentobarbital, and tenotomy of the antagonists (TA and EDL) of left soleus was performed additionally. The EMG and neurogram were recorded next day following the complete
recovery from the anesthesia. Tenotomy of the left soleus itself was further performed in the anesthetized rats. The final recordings of neuromuscular activities were performed in the same way on the next day. All recordings on the floor were performed keeping the ankle joint at dorsiflexed position with manipulation, if necessary. Although the rats did not appear to favor the affected leg, the ankle joint was dorsiflexed following the tenotomy of MG, LG, and Pi. The rats could not dorsiflex the ankle joint by themselves after additional tenotomy of TA and EDL followed by that of soleus. At the end of experiment, spinal transection at the L3 and L6 segmental levels including dorsal and ventral roots were performed and these neuromuscular activities were recorded to detect the baseline levels.

The locations of electrodes for EMGs and neurograms were also checked.

Effects of repeated anesthesia and surgeries on the EMGs and neurograms were also investigated. Responses of neuromuscular activities to each experimental condition were also studied using one rat for each situation. In this investigation, the responses of EMGs and neurograms were studied after a single surgery (either tenotomy of MG, LG, and Pi, tenotomy of MG, LG, Pi, TA, and EDL, or tenotomy of MG, LG, Pi, TA, EDL, and soleus). The baseline levels were also checked by spinal transection at L3 segmental level. The data obtained were identical to the results from the rats with multiple surgeries, suggesting that clear effects of multiple treatments, stress, or overloading were not observed. However, the amplitudes and patterns of EMGs and neurograms were not exactly the same for each situation and this may be due to the slight differences in the location of electrodes, which result in different sensitivity, for example. Therefore, the data repeatedly obtained from the same rats were used to make the effects of electrodes constant and to correlate the responses to each experimental condition. Further, only one day was allowed for recovery from the surgery to avoid effects of sarcomere remodeling. It was reported that sarcomere length, which was shortened after division of the proximal tendon, was normalized 7 days after tenotomy (15).
Effects of repeated anesthesia and multiple surgeries on body weight and daily food intake were also studied (Table 1). No treatments were performed on the day before the electrodes implantation. The control rats were also anesthetized by i.p. injection of sodium pentobarbital (5 mg/100 g body weight), although no surgeries were performed and they were not used for the analyses of EMGs and neurograms in the current investigation. The rats generally aroused within ~2 hours after the anesthesia. It was indicated that the body weight and the amount of food intake were not reduced in response to repeated anesthesia and surgeries during the experimental period, although significant growth-related gains were not seen either.

Analyses of data. The electrical signals were amplified (x1000) and recorded on a digital audio recorder (PC216AX, SONY, Tokyo) at 2.5 kHz. The amplified raw signals stored in the cassette tape recorder, in which the 60-Hz signals were filtered, were processed by a PowerLab/16sp (ML795, AD Instruments Inc., Australia), an analog-to-digital (A/D) converter, digitized at 2 kHz, and were stored on disk (Apple, PowerBook G3 computer). The total integrated areas of EMGs and neurograms throughout a series of experiments (before, during, and after hindlimb suspension) were determined using a computer software package (Chart v4.0.1, AD Instruments Inc., Australia). The total mean integrated neural activity per second was calculated (9).

Experiment 3: Neuromuscular activities during 2 weeks of hindlimb suspension

The EMG of left soleus and afferent and efferent neurograms were recorded during 2 weeks of continuous hindlimb suspension (n=10). Electrode implantation for EMG and neurogram was performed as mentioned above. After 2 days of complete recovery from the surgery, a sticky thick tape (~5 mm width and 3 cm length) with good cushion was placed longitudinally on the dorsal and ventral sides of the mid-tail of the rats. These tapes were
further surrounded cross-sectionally by a tape. Such treatment was performed loosely in order to keep the blood flow intact. A string was inserted through the gap between the tail and tape and fastened to the roof of cage at a height allowing the forelimbs to support the weight, yet prevent the hindlimbs from touching the floor or the wall of the cage. The rats could reach the food and water freely by using their forelimbs.

Before the initiation of hindlimb suspension, soleus EMG and afferent and efferent neurograms in conscious rats were recorded at quadrupedal posture on the floor for 2 hour. The integrated EMG level throughout the 2-hour period was used as the pre-suspension control level. Even though rats moved occasionally, they were generally sedentary. Subsequently, hindlimb suspension was started while the recordings were continued. The recordings of EMG and neurograms during suspension were performed for 8 consecutive hours during the light period everyday (8 a.m. – 4 p.m.). The raw signals were analyzed as described for the experiment 2. The total mean integrated neural activities per hour were calculated and the data were presented as the percentages relative to the pre-suspension control. The ranges of the ankle joint angle on the floor and during hindlimb suspension were also analyzed by video filming.

Experiment 4: Relationship between muscle fiber length and tension development

Muscle fiber length and tension development. The effects of changes in the ankle joint angle on the length of soleus muscle fibers and both inherent (passive) tension of an relaxed (inactivated) muscle under anesthesia and in vivo (active) tension developed by conscious rats were analyzed in rats before and after 14-day hindlimb suspension (n=5 each). The left soleus muscle was carefully exposed. A force transducer made of golden buckle with strain gage was placed at the distal tendon of soleus muscle. The tension at either 30°, 90°, 120°, 140°, or 160° of ankle joint angle in anesthetized rats was measured using a strain meter (PCD-30A, Kyowa, Tokyo, Japan) calibrated using an isometric force transducer (TB-654T,
Nihon Kohden, Tokyo, Japan). The knee angle does not influence the soleus length, but all measurements were performed keeping the knee angle at ~90°. After the rats aroused, the development of *in vivo* tension was also recorded at rest on the floor with or without body movement, for example, for postural adjustment.

The sarcomere number per fiber was also measured in the same rats. After the determination of tension development, the contralateral soleus muscle was removed, cleaned of excess fat and connective tissue, and was weighed immediately. The muscle was, then, carefully torn into longitudinal muscle fiber segments under the microscope, and the middle (longest) segment was stored in cellbanker (Nihon Zenyaku, Tokyo, Japan) at –80°C until analyzed.

The muscle fiber segments stored in the cellbanker were instantly thawed at 35°C. Collagens were digested in Dulbecco’s Modified Eagle’s Medium (DMEM, Invitrogen, CA, U.S.A.) containing 0.2 % type I collagenase, 0.2 % type IV collagenase, 1 % antibiotics, and 10 % new-born calf serum for 4 hours at 35°C. The collagenase-treated segments were fixed in 4 % buffered-formaldehyde for 30 minutes. Whole single muscle fibers, sampled from tendon-to-tendon, were isolated using fine needles. They were carefully collected by using pipette to avoid scratching the fibers and 60 fibers per muscle were mounted on a slide glass with coverslips with “struts” of hardened nail polish on the corners to minimize fiber compression. Working solution of both type I and IV collagenase was gel-purified to remove the clostripain, which supposedly strips the basal lamina of the fibers (3).

A Fluoview confocal microscope with an argon laser (488 nm of mean wavelength, Olympus, Tokyo, Japan) was used to analyze the muscle fiber and sarcomere length. The fiber length and the length of 10 consecutive sarcomeres, randomly chosen from three non-overlapping regions along the fiber length, were measured in each fiber by Nomarski optic scan using calibrated measurement software (Olympus, Tokyo, Japan). The levels of fiber length were calculated by multiplying the measured fiber length by 2.5 µm and then
dividing by the measured single sarcomere length. Further, the total sarcomere number per fiber was also calculated.

The mean length of sarcomere at a certain ankle joint angle. The in vivo mean length of sarcomere at a certain ankle joint angle was also measured in the soleus muscle fibers with or without 14 days of hindlimb suspension (n=10 each). The whole hindlimbs were isolated from both sides and submerged in 4% buffered-formaldehyde keeping the anterior angle of ankle joint at either ~30°, 120°, 140°, or 160° (n=5 for each angle). The soleus muscle was removed after 30 minutes. Subsequently, single muscle fibers were isolated from middle portion of the muscle using fine tweezers under microscope. Thirty fibers per muscle were mounted on a slide glass with coverslip with “struts” of hardened nail polish on the corners to minimize fiber compression. The length of 10 consecutive sarcomeres was measured in three different sites and the mean sarcomere length was calculated.

Statistical analyses

All data were presented as mean ± SEM. The EMGs and neurograms (mV/sec) were used for analyses in the experiment 2. In the experiment 3, the percent changes of neuromuscular activities relative to those recorded during normal ground support before suspension were compared. In the experiment 4, the level of passive tension development at a given ankle joint was compared between the soleus muscle with or without 14 days of hindlimb suspension. Further, the mean sarcomere length at a given ankle joint obtained from 30 fibers for each muscle (n=5) was also compared between these groups (10 rats per each group). The effects of 14 days of hindlimb suspension on the mean sarcomere number per whole fiber were studied using the mean value obtained from 60 fibers for each muscle (n=5). Statistical significance was examined by repeated measures of ANOVA followed by Scheffé’s post hoc test in the experiment 2 and 4 (passive tension production and sarcomere length). Sarcomere number per fiber in experiment 4 was compared by unpaired t-test.
Paired *t*-test was used to compare with the respective control in the experiment 3.
Differences were considered significant at the 0.05 level of confidence.

**RESULTS**

*Experiment 1: Motoneuron labeling*

Figure 1 shows the location of nuclear yellow-labeled motoneurons in the longitudinal section from the L4 through L6 segmental levels of the rat spinal cord. Although the motoneurons innervating the specific muscle were labeled in 5 rats for each muscle, the location of motoneurons were distributed in the same area in all rats. Therefore, the distribution in one rat is illustrated in the figure. Motoneurons associated with ankle plantarflexors (soleus, Pl, MG, and LG) and dorsiflexors (TA and EDL) were distributed between the L4 and L5 segmental levels of the spinal cord. Motoneurons innervating EDL, TA, and Pl were distributed between the middle of L4 and the middle of L5 segment. Motoneurons innervating LG were distributed between the lower L4 and lower L5 segment. Further, the motoneurons associated with MG and soleus were observed mainly at the L5 segment.

*Experiment 2: Effects of hindlimb muscle activities on the neurograms*

The activity levels of afferent and efferent neurograms decreased ~37% and 20% following an acute hindlimb suspension, respectively, when the tendons of all hindlimb muscles were intact (Figs. 2 and 3, *p*<0.05). However, those activities were normalized after the termination of the hindlimb suspension. Soleus EMG activity decreased in response to an acute unloading to ~12% of the level at quadrupedal posture on the floor (*p*<0.05). Its activity was restored when the muscles were loaded again. The EMG activities of MG and TA did not significantly change during an acute hindlimb suspension.

After tenotomy of MG, LG and Pl, the level of afferent neurogram recorded at
quadrupedal posture on the floor increased ~70% compared with that before tenotomy (Figs. 2 and 3, p<0.05). Although it was decreased ~53% from the level on the floor in response to an acute unloading (p<0.05), the afferent neurogram returned to the pre-suspension level when the hindlimb suspension was terminated. The levels of efferent input were not significantly affected by tenotomy of soleus synergists and unloading. The EMG activity of soleus recorded at quadrupedal posture on the floor did not significantly change after the tenotomy of synergists, but decreased (84%) in response to an acute unloading (p<0.05). The soleus EMG immediately recovered to pre-suspension level when the muscle was loaded on the floor again. The EMG activity of MG at quadrupedal posture on the floor decreased to 61% of the pre-tenotomy level following the tenotomy of MG itself. The EMG activity of MG, in which the distal tendon was tenotomized, did not respond to hindlimb suspension and reloading on the floor. The TA EMG was not affected by tenotomy of plantarflexors.

The afferent neurograms that were recorded when the rat was on the floor decreased toward the control level (before tenotomy of any muscles), after further tenotomy of TA and EDL in addition to MG, LG, and Pl (Figs. 2 and 3, p>0.05). The afferent neurogram was lowered ~62% in response to hindlimb suspension (p<0.05) and returned to pre-suspension level when the hindlimbs were returned on the floor. The level of efferent neurogram was not influenced at all. Soleus EMG recorded during quadrupedal posture on the floor did not change by the additional tenotomy of TA and EDL. However, it was still inhibited in response to suspension (~86%, p<0.05). The EMG activities of MG and TA were similar to the levels observed after tenotomy of MG, LG, and Pl.

Finally, the activity levels of afferent and efferent neurograms and soleus EMG during quadrupedal posture on the floor were decreased ~63 (p<0.05), ~27 (p>0.05), and ~91% (p < 0.05), when the distal tendon of soleus itself was tenotomized (Figs. 2 and 3). None of these levels were influenced by hindlimb suspension and reloading on the floor. The EMG activities of MG and TA were not affected either.
The baseline levels of these neuromuscular activities are also shown in Figure 3. All of the neuromuscular activities were reduced after the spinal transection at the L₃ and L₆ segmental levels including dorsal and ventral roots. Although the soleus EMG and afferent neurogram decreased after hindlimb suspension or tenotomy of soleus as was stated above, those values were still greater than the baseline levels by 300% and 23%, respectively. The lowest levels of EMGs in MG and TA and efferent neurogram were 126%, 241%, and 73% greater than the baseline activities, respectively. All of the values shown in Figure 3 were significantly greater than the baseline levels, indicating that the data shown above reflect the actual neuromuscular activity levels.

Further, a significant positive correlation was observed between the integrated soleus EMG and afferent neurogram (Fig. 4), suggesting that the afferent neurogram recorded at the L₅ segmental level of spinal cord may generally reflect the activity of soleus muscle. Some neurogram activity, which could be originated from tissue other than soleus, was also noted when soleus EMG was silent after tenotomy of soleus itself (Fig. 2, bottom-right panel). However, the patterns of responses were generally similar for EMG and afferent neurogram. Significant correlation was not observed between the levels of soleus EMG and efferent neurogram (r=0.36, p=0.60).

**Experiment 3: Neuromuscular activities during 2 weeks of hindlimb suspension**

Soleus EMG activity decreased immediately following hindlimb suspension to ~12% of the pre-suspension level on the floor (Fig. 5). Even though the lower EMG levels were maintained during 4 days, the level gradually increased thereafter and reached the pre-suspension level after 14 days. The afferent neurogram decreased ~37 % from the pre-suspension level after hindlimb suspension (p<0.05). The lower activities (60-68% of the pre-suspension level) were maintained during 8 days of suspension. However, the activity gradually increased after the 9th day and reached to the levels even greater than
before suspension (p>0.05). The efferent neurogram also decreased immediately following suspension (~20%, p<0.05). Generally, the level was maintained low up to the 8th day, but was elevated to ~125% and 130% of pre-suspension level after 10 days of suspension (p < 0.05). The recordings of EMG and neurograms during suspension were performed for 8 consecutive hours during the light period everyday (8 a.m. – 4 p.m.) as was explained the Experimental procedures.

The ranges of the ankle joint angle on the floor and during hindlimb suspension were ~30-90° and ~90-160°, respectively. Higher EMG levels of soleus were maintained when the ankle joint angle was maintained between ~30-90° on the floor. However, those levels were, on the contrary, reduced once the ankle joints were plantarflexed following an acute hindlimb suspension of rat. The EMG activities at ~90° of ankle joint on the floor and during suspension were completely different. Even though the degree of ankle joint was approximately identical at ~90°, the EMG level was significantly less during hindlimb suspension due to the lack of external load.

Experiment 4: Relationship between muscle fiber length and tension development

In the control rats, the critical level of ankle joint angle for passive tension development was ~120°. No passive tension was detected when the angle of ankle joint was greater than 120°. The mean levels of passive tension at 90° and 30° ankle joint angle were 16.4 and 43.6 g, respectively. The critical angle of ankle joint for development of passive tension after 14 days of hindlimb suspension was ~140°. The mean passive tensions detected at 120°, 90°, and 30° ankle joint angle were 2.5, 7.0, and 21.5 g, respectively. The tensions developed at 90° and 30° ankle joint angle after suspension were significantly less than those before suspension (p<0.05), may be due to muscle atrophy. Mean muscle weight of the suspended group was ~43% less than the pre-suspension level (p<0.05, Data not shown). The arrows in the figure indicate that some degrees of active tensions were detected, when EMGs were
present in aroused rats. The active tension in the pre-suspension controls was not detected at 160° and 140°. Some degrees of development were noted at 140°, but not at 160°, in the suspended group. However, the levels of active tension development and EMG were variable, because the muscle contraction was voluntary.

The mean sarcomere lengths at 30°, 120°, 140°, and 160° ankle joint angle in control rats were 3.03, 2.16, 2.05, and 2.05 μm, respectively (Fig. 6B). The sarcomeres were passively stretched at 30° and the mean length was greater than 2.5 μm. And the sarcomeres were shortened at 120° (p<0.05). The mean length was further reduced due to hyperextension of ankle joint (p<0.05), even though the values at 140° and 160° were identical. The mean sarcomere length at a given ankle joint angle was increased after 14 days of hindlimb suspension. The length at 120° and 30° ankle joint angle was significantly longer than that of the pre-suspension level (p<0.05). This phenomenon was related to the decreased sarcomere number (Fig. 6C) and muscle fiber length (Fig. 7). The mean sarcomere number per fiber was 3,869 relative to 5,331 in the pre-suspension control (Fig. 6C, p<0.05). The distribution of muscle fibers with various lengths is shown in Figure 7. Shorter fibers were noted in the post-suspension group. The mean lengths before and after 14-day suspension were 13.3±0.2 and 9.7±0.2 mm, respectively (p<0.05).

The distribution of muscle fibers with various mean sarcomere lengths is illustrated in Figure 8. Since plantarflexion of ankle joint at 160° caused passive shortening of sarcomeres as is shown in Figure 6B, the mean length of all fibers analyzed was less than 2.4 μm in the pre-suspension group and the mean length was 2.05 (±0.02) μm. However, fibers with longer sarcomeres were noted in the post-suspension group and the mean length was 2.15 (±0.03) μm.

**DISCUSSION**

Acute and chronic responses of EMG in hindlimb muscles and both afferent and
efferent neurograms, recorded at the L5 segmental level of the spinal cord, to hindlimb suspension and/or tenotomy were investigated in adult rats. To our knowledge, this is the first study showing the relationship between the EMG, neurogram, sarcomere length, and tension development of soleus in response to gravitational unloading.

*Distribution of motoneurons innervating soleus:*

Motoneurons innervating major hindlimb muscles were distributed between the L4 and L5 segmental levels. Especially, those innervating soleus, MG and LG were observed at the L5 segmental level, where the neurograms were recorded. This observation generally agrees with the previous studies (16, 27). Therefore, the neurogram activities recorded at the L5 segmental level of the spinal cord may reflect the activity patterns of MG and LG. However, the afferent neurogram recorded at quadrupedal posture on the floor was even increased significantly, when MG and LG, as well as Pl, were tenotomized. The level of EMG in MG, on the contrary, was significantly decreased by tenotomy of MG. It is suggested that soleus muscle was overloaded in response to the tenotomy of the synergists, since soleus EMG was also increased (~16%, p>0.05). The afferent input was inhibited, when the soleus EMG was decreased after tenotomy of soleus itself. The patterns observed in response to various treatments were similar for the afferent neurogram and soleus EMG with a significant positive correlation. However, the patterns observed in MG EMG were completely different and the absolute activity level of MG EMG at quadrupedal posture on the floor in control situation was 87% less than that of soleus. These results suggest that the level of afferent neurogram recorded at the L5 segmental level of the spinal cord may reflect the activity level of soleus mainly.

*Acute responses of neuromuscular activities:*

Responses to unloading: The levels of soleus EMG and afferent neurogram decreased
following acute hindlimb suspension of rats in the present study. The decrease of soleus EMG in response to hindlimb suspension agrees with the results observed elsewhere (1, 19, 20, 24, 26, 28). We also reported that both soleus EMG and afferent neurogram recorded at the L5 segmental level of the spinal cord of rat were increased gradually when the gravity level was increased from 1-g to 2-g during the ascending phase of a parabolic flight of a jet airplane (12). But they were suddenly decreased, when the rat was exposed to μ-g. Those levels were maintained low during the 20-second exposure to μ-g environment.

Hindlimb suspension and exposure to μ-g generally cause plantarflexion of ankle joints, which then results in passive shortening of ankle plantarflexors, such as soleus (12, 19). The tension development of soleus was inhibited when the lengths of muscle, muscle fibers, or sarcomeres were shortened in response to acute hindlimb suspension. The results in the present investigation suggested that the suspension-related decreases in EMG and afferent neurogram were closely associated with the passive reduction of sarcomere length and inhibition of tension development caused by plantarflexion of ankle joints.

The levels of soleus EMG and afferent neurogram during hindlimb suspension were not influenced significantly by additional tenotomy of synergists and/or soleus itself. Ohara et al. (18) reported that suspension-related atrophy of soleus muscle was not promoted when tenotomy of ankle extensors, denervation, or both tenotomy and denervation were performed in addition to hindlimb suspension. These data suggest that the decreases of EMG and afferent neurogram associated with the passive shortening of soleus, which inhibits tension development and afferent input, reach the maximum levels by plantarflexion of ankle joint alone during hindlimb suspension.

The precise mechanism responsible for the greater increase in the afferent neurogram than EMG level in response to tenotomy of synergists is unclear. But one possibility might be that the mechanical stretch due to overloading may increase the discharges of Ia and II fibers (6). Further, the results from our recent study showed that the growth-related increase
of soleus muscle of rats was not enhanced significantly by exposure to 2-g environment during the postnatal day 4 and month 3, relative to the growth of the cage-control rats (32). It was speculated that the load on the soleus muscle during centrifugation at 2-g may be greater than that at 1-g environment, but the effects on soleus may be minor if the rat is sedentary. If a similar ankle joint angle is maintained at a fully dorsiflexed position, soleus length is identical regardless of the environmental gravity level.

The level of efferent neurogram decreased following acute hindlimb suspension in the present study. However, it was not influenced significantly by tenotomy of synergists and/or soleus itself, in general. Efferent neurogram, as well as the afferent neurogram and EMG, decreased after spinal transection at the L₃ and L₆ levels. It is indicated that efferent neurogram recorded at the L₅ segmental level does not necessarily reflect the neural input associated with activity of soleus predominantly, even though the neurons that innervate soleus distributed at that segmental level. It is also indicated that the neurons distributed at the L₅ may also innervate other muscles and/or tissues. For example, the EMG activity of MG did not change and that of TA was even increased slightly (p>0.05) in response to unloading, as was mentioned above (12, 18, 22). Thus, the responses of soleus EMG and the efferent neurogram recorded at the L₅ may not be correlated.

Responses to loading: The dorsiflexion of ankle joints at ~30° on the floor caused a passive stretching of soleus fibers with mean sarcomere length of 3.03 µm and active EMG was maintained in the pre-suspension control rats. The level of afferent neurogram at quadrupedal posture on the floor was also significantly increased 1.7 folds after functional overload for soleus, relative to the pre-tenotomy control level. Further, these neural activities were increased when the rats were exposed to hypergravity during the ascending phase of parabolic flight (12) as was stated above, suggesting that the responses of these neuromuscular activities are load-dependent. Development of both passive and active
tension was noted in muscles at dorsiflexed position, suggesting that the muscles were loaded. The soleus EMG on the floor was further increased (~16%, p>0.05), when additional load was applied by the tenotomy of the synergists (MG, LG, and PL). Soleus EMG and afferent neurogram during hindlimb suspension were also increased, if soleus muscle was stretched by joint immobilization at a dorsiflexed position (unpublished observation).

**Chronic responses of neuromuscular activities:**

Decreased levels of soleus EMG and afferent and efferent neurograms were generally maintained low up to the 4th day during hindlimb suspension. Although Blewett and Elder (4) reported that the EMGs of both soleus and PL were maintained low during 28 days of suspension, the soleus EMG level was gradually recovered during suspension in the current study as was observed previously (1, 19, 26). Blewett and Elder (4) analyzed the number and amplitude of turns and they checked the body weight every 4th day of hindlimb suspension. We analyzed the integrated area of EMG and hindlimb suspension was continuous. The level of soleus EMG also remained low during the first 4 days of suspension even in our study. The ground support activity performed every 4 day in the study by Blewett and Elder (4) may be one of the causes for the inhibition of EMG recovery.

Alford et al. (1) also reported that the soleus EMG activity was recovered during suspension. Generally, voluntary activity level of rat on the floor is increased during dark period (23). However, typical differences were not observed in the EMG levels during light and dark period (1). Therefore, the changes in the total activity during 24-hour period were compared, and the level was increased to the pre-suspension level within 14 days of suspension. These results may suggest that the neuromuscular activities during hindlimb suspension were restored during both light and dark period, although those levels were analyzed only during the light period in the current study.

It was reported that immobilization of skeletal muscle in stretched or shortened position
induces increase or decrease of sarcomere numbers (29, 30, 33). Shah et al. (29) reported that sarcomere numbers in soleus muscle fibers were reduced after 28 days of immobilization of the ankle joint at a maximally plantarflexed position. The joints were not immobilized in the present study, but the ankle joints were kept plantarflexed at 90°–160° during suspension. Passive tension development of the soleus muscle was decreased in response to plantarflexion of ankle joint (Fig. 6A), which has a direct influence on the muscle length (12, 19). Even though the tension development was still very low when the ankle joint angle was 160°, it became capable at 140° of ankle joint after 14 days of suspension (Fig. 6). Further, the mean level of EMG, as well as afferent input, during hindlimb suspension after 14 days of suspension was generally identical to that of cage control, even if the normal ground support is still inhibited and external gravitational load is essentially zero.

It was clear that the number of sarcomeres was decreased in response to chronic shortening of the muscle length and sarcomere length at a given angle of ankle joint was increased. Therefore, the soleus muscle fibers were slightly stretched even though the ankle joints were still plantarflexed during suspension. The mean sarcomere lengths at ~60° on the floor before suspension and at ~90° during suspension after 14 days of unloading were similar, suggesting that force could be produced due to the suspension-related sarcomere remodeling even without an external load.

It was also shown that the discharges of Ia and II fibers in response to a given stretch of rat soleus muscle fibers were increased after 14 days of hindlimb suspension, suggesting that increased connective tissues could contribute to a better transmission of passive mechanical stretch to muscle spindles (6). Increased afferent discharges may also contribute to the elevated efferent neurogram. Further, effects of gravitational unloading on the elasticity of muscle fibers were recently studied elsewhere. The relative proportion of type III collagen, which is more elastic than type I, was increased in response to 14 and 28 days of hindlimb suspension of rats (17). Toursel et al. (31) reported that passive tension of soleus fibers,
atrophyed after 14 days of hindlimb suspension, increased less steeply in response to stretch than that of control fibers. Although they concluded that such phenomenon may be due to the decreased amount of connectin, reduction of fiber diameter (~54 and 47% in slow and fast fibers, respectively) could be another factor. However, Goto et al. (8) reported that elasticity of connectin (titin) filaments in the I-band region of atrophied soleus muscle fibers was reduced following hindlimb suspension. The recovery of neuromuscular activities during hindlimb suspension may be related to these phenomena.

In conclusion, responses of EMG in soleus and both afferent and efferent neurograms at the L5 segmental level of spinal cord were investigated during acute and chronic unloading in adult rats. It was suggested that the level of the afferent, not always the efferent, neurogram recorded at the L5 segmental level reflects the neural input associated with the activity level of soleus predominantly. The EMG and neurograms remained low up to the 4th day, but recovered to the pre-experimental levels within 14 days, due to reorganization of sarcomere number and length, as well as the shortening of muscle fiber length and increased tension development. It is indicated that the levels of EMG and afferent neurogram associated with antigravity muscle are closely related to the tension development of the muscle. It is further suggested that force can be produced without an external load.

ACKNOWLEDGEMENTS

This study was carried out as a part of “Ground-based Research Announcement for Space Utilization” promoted by Japan Space Forum, Tokyo, Grant-in-Aid for Scientific Research (A, 15200049) from Japan Society for the Promotion of Science, and Grant-in Aid for Young Scientists (B, 15700417) from Ministry of Education, Culture, Sports, Science and Technology.
REFERENCES


10. **Ishihara A, Ohira Y, Roy RR, Nagaoka S, Sekiguchi C, Hinds WE, and Edgerton VR.** Influence of spaceflight on succinate dehydrogenase activity and soma size of rat ventral


29. **Shah SB, Peters D, Jordan KA, Milner DJ, Friden J, Capetanaki Y, and Lieber RL.** Sarcomere number regulation maintained after immobilization in desmin-null mouse


Table 1

The changes in the body weight and the amount of food intake during the experiment 2.

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<th>4th</th>
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Mean±SEM.  n=5 in each group.  Pl, plantaris; MG, medial gastrocnemius; LG, lateral gastrocnemius; TA, tibialis anterior; EDL, extensor digitorum longus.
FIGURE LEGENDS

Figure 1: The location (upper illustration) and typical pattern (bottom picture) of nuclear yellow-labeled motoneurons in the longitudinal section of the rat spinal cord. The numbers of labeled motoneurons in one rat are shown in the parentheses. Total numbers of motoneurons detected in 5 rats are 168, 269, 236, 313, 197, and 164 for EDL, MG, TA, LG, Pl, and soleus, respectively. The numbers 4-6 indicate the segmental levels of the spinal cord. EDL, extensor digitorum longus; MG, medial gastrocnemius; TA, tibialis anterior; LG, lateral gastrocnemius; Pl, plantaris. The arrows in the bottom picture indicate the nuclear yellow-labeled motoneurons. WM, white matter; GM, gray matter; R, rostral direction; M, medial direction. Scale bar = 100 µm.

Figure 2: Typical patterns of afferent and efferent neurograms recorded at the L5 segmental level of the spinal cord and electromyogram (EMG) activities of soleus, medial gastrocnemius (MG), and tibialis anterior (TA) during quadrupedal posture on the floor and 20-second of hindlimb suspension before tenotomy (upper-left), after tenotomy of MG, LG and Pl (upper-right), of MG, LG, Pl, TA and EDL (lower-left), and of MG, LG, Pl, TA, EDL and soleus (lower-right). LG, lateral gastrocnemius; Pl, plantaris; EDL, extensor digitorum longus.

Figure 3: The integrated levels of afferent (A) and efferent (B) neurograms and electromyogram (EMG) of soleus (C), medial gastrocnemius (D) and tibialis anterior (E) during quadrupedal posture on the floor and 20-second of hindlimb suspension in 5 rats. Mean ± SEM. *, §, †, ‡, and ¶: Significantly different from the level before hindlimb suspension (on the floor, pre) among the respective group indicated by the specific bar, during hindlimb suspension among the respective group indicated by the
specific bar, control (before tenotomy, white bars), after tenotomy of MG, LG and Pl (grey bars), and of MG, LG, Pl, TA and EDL (black bars), respectively (p<0.05). The baseline levels of these neuromuscular activities are shown by the horizontal broken lines. The baseline activities were checked after the spinal transection at the L3 and L6 segmental levels, including dorsal and ventral roots, after the end of experiment. All of the values shown in the figure were significantly greater than the baseline activities (p<0.05). MG, medial gastrocnemius; LG, lateral gastrocnemius; Pl, plantaris; TA, tibialis anterior; EDL, extensor digitorum longus.

Figure 4: The relationship between the integrated levels of soleus electromyogram (EMG) and afferent neurogram at the L5 segmental level of the spinal cord. MG, medial gastrocnemius; LG, lateral gastrocnemius; Pl, plantaris; TA, tibialis anterior; EDL, extensor digitorum longus.

Figure 5: Changes in the integrated levels of afferent (open circle) and efferent (closed circle) neurograms and soleus electromyogram (EMG, gray bar) before and during 14-day hindlimb suspension in 10 rats. Mean ± SEM. *: Significantly different from the pre-suspension level recorded at quadrupedal posture on the floor (p<0.05).

Figure 6: The relationship between the anterior angle of ankle joint and passive and active tension development (A, n=5 for each group) and the mean in vivo length of sarcomeres (B, 10 rats for each group and 5 muscles or 5x30 fibers for each angle) before and after 14-day hindlimb suspension. The total sarcomere number per fiber is also illustrated (C, 5 muscles or 5x60 fibers for each group). Mean ± SEM. * and †: Significantly different from the pre-suspension control and the levels at 160° ankle joint angle (p<0.05).
Figure 7: The distribution of soleus muscle fibers with various lengths before and after 14 days of hindlimb suspension. Five muscles or 5x60 fibers for each group.

Figure 8: The distribution of soleus muscle fibers with various sarcomere lengths at 160° of ankle joint angle before and after 14 days of hindlimb suspension. Five muscles or 5x30 fibers for each group.
Figure 1
Figure 2

Control (before tenotomy)  Tenotomized (MG, LG and PI)

Afferent neurogram
Efferent neurogram
Soleus EMG
MG EMG
TA EMG

Tenotomized (MG, LG, PI, TA and EDL)

Afferent neurogram
Efferent neurogram
Soleus EMG
MG EMG
TA EMG

On the floor  Hindlimb suspension (20 seconds)  On the floor

On the floor  Hindlimb suspension (20 seconds)  On the floor

0.02 mV
0.02 mV
0.2 mV
0.02 mV
0.02 mV

0.02 mV
0.02 mV
0.2 mV
0.02 mV
0.02 mV
Figure 3

A  Afferent neurogram

B  Efferent neurogram

C  Soleus EMG

D  MG EMG

E  TA EMG

Legend:
- Control (before tenotomy)
- Tenotomized (MG, LG, and PI)
- Tenotomized (MG, LG, PI, TA, and EDL)
- Tenotomized (MG, LG, PI, TA, EDL, and Soleus)
- Baseline activities
Figure 4

The figure shows a scatter plot with the relationship between the Soleus EMG (mV s⁻¹) and the Afferent neurogram (mV s⁻¹). The equation of the best fit line is given as:

\[ y = 0.08x + 2.30 \]

With a correlation coefficient (r) of 0.90 and a p-value of <0.05. Different symbols represent different conditions:
- ○: On the floor (before tenotomy)
- ●: Hindlimb suspension (before tenotomy)
- △: On the floor (MG, LG, and PL tenotomized)
- ▲: Hindlimb suspension (MG, LG, and PL tenotomized)
- □: On the floor (MG, LG, PL, TA, and EDL tenotomized)
- ■: Hindlimb suspension (MG, LG, PL, TA, and EDL tenotomized)
- ✫: On the floor (MG, LG, PL, TA, EDL, and soleus tenotomized)
- ★: Hindlimb suspension (MG, LG, PL, TA, EDL, and soleus tenotomized)
Figure 5

![Chart showing changes in Soleus EMG, Afferent neurogram, and Efferent neurogram over days of hindlimb suspension.]
Figure 6

A. Passive tension production (g)

B. Sarcomere length (µm)

C. Sarcomere number per fiber

On the floor
Hindlimb suspension
Figure 7
Figure 8

[Histogram showing percent distribution of fibers at hip and ankle joints for pre-suspension and post-suspension conditions.]