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太治野 純一

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Intermittent application of hypergravity by centrifugation attenuates disruption of rat gait induced by 2 weeks of simulated microgravity

Junichi Tajino^a, Akira Ito^a, Momoko Nagai^a, Xiangkai Zhang^a, Shoki Yamaguchi^a,
Hirotaka Iijima^a, Tomoki Aoyama^b, Hiroshi Kuroki^{a*}

^a Department of Motor Function Analysis, ^b Department of Development and Rehabilitation of Motor Function, Human Health Sciences, Graduate School of Medicine, Kyoto University, 53 Shogoin Kawahara-cho, Sakyo-ku, 606-8507, Kyoto, Japan

Authors' e-mail addresses:

tajino.junichi.57z@st.kyoto-u.ac.jp, ito.akira.27n@st.kyoto-u.ac.jp,
nagai.momoko.36v@st.kyoto-u.ac.jp, zhang.xiangkai.48v@st.kyoto-u.ac.jp,
yamaguchi.shouki.26n@st.kyoto-u.ac.jp, ijima.hirotaka.75s@st.kyoto-u.ac.jp,
aoyama.tomoki.4e@kyoto-u.ac.jp, kuroki.hiroshi.6s@kyoto-u.ac.jp

*Corresponding author: Hiroshi Kuroki

Department of Motor Function Analysis, Human Health Sciences,
Graduate School of Medicine, Kyoto University,
53 Shogoin Kawahara-cho, Sakyo-ku, 606-8507, Kyoto, Japan

Tel: +81-75-751-3963; Fax: +81-75-751-3909

E-mail: kuroki.hiroshi.6s@kyoto-u.ac.jp

Abstract

The effects of intermittent hypergravity on gait alterations and hindlimb muscle atrophy in rats induced by 2 weeks of simulated microgravity were investigated. Rats were submitted to hindlimb unloading for 2 weeks (unloading period), followed by 2 weeks of reloading (recovery period). During the unloading period, animals were subjected to the following treatments: (1) free in cages (Control); (2) continuous unloading (UL); (3) released from unloading for 1 hour per day (UL+1G); (4) hypergravity for 1 h per day using a centrifuge for small animals (UL+2G). The relative weights of muscles to the whole body weight and kinematics properties of hindlimbs during gait were evaluated. UL rats walked with their hindlimbs overextended, and the oscillation of their limb motion had become narrowed and forward-shifted after the unloading period, and this persisted for at least 2 weeks after the termination of unloading. However, these locomotor alterations were attenuated in rats subjected to UL+2G centrifugation despite minor systematic changes in muscle recovery. These findings indicate hypergravity application could counteract the adverse effects of simulated or actual microgravity environments.

Keywords: Hindlimb unloading; Centrifugation; Rats; Locomotion; Sensorimotor adaptation

Abbreviations

ASt, ankle angle at mid stance

CO, center of oscillation

KSt, knee angle at mid stance

MG, medial gastrocnemius

MTP, 5th metatarsophalangeal joint

RO, range of oscillation

Sol, soleus

UL, unloaded

UL+1G, unloaded +1 gravity application (normal gravity)

UL+2G, unloaded +2 gravity application (twice normal gravity [hypergravity])

1. Introduction

Exposure to microgravity environments induces multiple alterations in sensorimotor apparatuses, necessitating an adaptation to such unfamiliar environments. Although such alterations involve muscular and skeletal properties [1,2], recent studies show that exposure to microgravity environments alters the nervous system [3,4], behavior [5,6], and kinematics properties as well [7–9]. Regarding locomotion, rats exposed to microgravity exhibit altered motion characterized by hyperextension of the knee and ankle joints during stance phase, which is described as “walking on its toes” [8], hypodynamia, and forward-shifted motion of the hindlimbs (i.e., less extension backward) [10–12] (Supplementary Fig. 1). Furthermore, exposure to altered gravity might irreversibly transform gait characteristics after termination of the perturbation [8,11,13,14]. Several countermeasures such as strength training, treadmill exercises, and lower-body negative pressure (LBPP) have been employed to reduce the adverse effects on musculoskeletal and kinematics properties induced by microgravity [15].

One such countermeasure is artificial gravity produced by a centrifuge, which could provide an effective solution, because most of the disturbances due to microgravity can be attenuated by a sufficient gravity load. However, to date, most of these studies investigating advantages of artificial gravity have been focused on musculoskeletal aspects [2,16,17], and little is known about the motion deficits. Further, regarding practical applications, the time that one can spare for daily countermeasures is limited [2,18]. Nevertheless, the effectiveness of intermittent centrifugation as a countermeasure against microgravity-induced deficiencies are not

well understood not only in musculoskeletal issues but also in motor alteration although Bouet et al. have investigated effects of chronic hypergravity on locomotion in rats by using artificial gravity (i.e., $2\times$ gravitational force: 2G) [14].

Therefore, in this study, we investigated the following: (1) whether intermittent application of hypergravity using centrifugation prevents the disruption of gait induced by 2 weeks of hindlimb unloading in rats; (2) whether the recovery of atrophy in hindlimb extensor muscles is associated with the gait alterations.

2. Materials and methods

We used 4 groups of 8-week-old male Wistar rats ($N=72$) following 1 week of training for treadmill walking. All animals were kept in the same temperature- and light-controlled room. The study protocol was approved by the Animal Experimentation Committee of the Graduate School of Medicine, Kyoto University. All experiments were conducted in accordance with the National Institutes of Health's Guidelines for the Care and Use of Laboratory Animals.

2.1. Treadmill acclimatization and grouping

One week prior to the experiments, all animals were trained to walk on a treadmill as previously described [8,19–22]. The rats were made to walk for 20 min at 20 cm s^{-1} [20] every other day during the acclimatization period. Subsequently, the rats were randomly distributed into 4 groups: (1) control (Control), (2) unloaded (UL), (3) unloaded +1G (UL+1G [normal gravity]), and (4) unloaded +2G (UL+2G [twice normal gravity]). As the Control group, rats were reared in regular cages and were

allowed to move freely throughout the 4-week experimental period. The other groups were submitted to hindlimb unloading for the first 2 weeks, followed by another 2 weeks of free movement (see sections below for details).

2.2. Hindlimb unloading

In the first 2 weeks of the experiment, the rats in the UL, UL+1G, and UL+2G groups were unloaded by their tail, and allowed to move freely on their forelimbs (unloading period). The hindlimb unloading was performed according to a modified procedure borrowed from Wronski and Morey-Holton [23,24]. The rats in the UL group were kept unloaded throughout the unloading period. Meanwhile, in the UL+1G group, the animals were relieved from unloading and placed on the ground 1 hour per day 6 times per week. In the UL+2G group, the animals were relieved from unloading and submitted to centrifugation at the same timing [2,18]. After the initial 2 week unloading period, the UL+1G and UL+2G groups were re-loaded and allowed to move freely for another 2 weeks (recovery period) until the final evaluation. Evaluations were performed every 2 weeks. Six animals were extracted at each time point of interest (0 week [beginning of the unloading period], 2 weeks [termination of the unloading period], 4 weeks [end of the recovery period]) and subjected to evaluation (Supplementary Fig. 2 for details).

2.3. Intermittent centrifugation

We used a centrifuge customized for small animals [25,26], with a 0.5-m-radius arm (Uchida Electron, Tokyo, Japan). Rotating the arm at 56 rpm generates 2G

artificial gravity in the resultant force line between the centrifugal force and vertical gravity. Animals in the UL+2G group were centrifuged while relieved from unloading. During centrifugation, they were placed in individual cages, each equipped with a small video camera to monitor their behavior and posture (Supplementary Video 1).

2.4. Kinematic analysis

At each time point of interest, 6 animals from each group were randomly selected and the kinematics properties of hindlimbs during ambulation on a treadmill moving at 20 cm s⁻¹ were assessed. The motion was captured at 120 Hz using a 3-dimensional (3-D) motion capture apparatus (Kinema Tracer System, Kissei Comtec, Nagano, Japan). This system consists of four CCD (charged coupled device) cameras (two of which are placed in line on both the right and left side of the treadmill), and of an image processor that allows reconstruction of 3-D movements from the captured movies (Supplementary Video 2). Before each capture session, colored hemispherical plastic markers (diameter: 0.3 cm), which correspond to 5 landmarks employed in order to detect joint displacements, were attached onto shaved skin while the animal was under light anaesthesia induced using isoflurane (Supplementary Fig. 3). The landmarks were as follows: the anterior superior iliac spine, trochanter major (i.e., hip), knee joint (knee), lateral malleolus (ankle), and the 5th metatarsophalangeal joint (MTP). Then, each rat walked on a treadmill moving at 20 cm s⁻¹. Although each recording session involved several trials until the animal performed successive gait, each bout lasted <10 s, and breaks for the subject were introduced to avoid fatigue. For subsequent analysis, a total of 10 steps for each animal were obtained from

portions of sequences in which the animal walked at a uniform velocity for at least 5 consecutive steps [22]. To ensure data accuracy, the precise coordinates were calibrated by recording a cube of known size ($5 \times 20 \times 10$ cm [$x \times y \times z$]) before each session. The coordination of the 3-D directions for the x -, y -, z -axes were lateral, anterior, and vertical, respectively (i.e., the right-hand rule: Supplementary Video 2, right panel).

After tracing the markers, joint displacements, which represent the kinematics properties, were automatically calculated by the system. The parameters were defined as follows: (1), the knee angle and (2), the ankle angle at stance phase (KSt and ASt, respectively): the angle of knee and ankle joint when the MTP marker was vertical with the hip marker in the y - z plane during the stance phase; (3), limb angle: the angle between the y -axis and the line connecting the hip and the ankle marker; (4), range of oscillation (RO): the difference in the limb angle between the paw contact and lift off; (5), center of oscillation (CO): the mid-point limb angle over the RO [8,27,28]. For instance, when the limb angle of the paw contact and lift off is 70° and 130° respectively, the CO is 100° . The smaller value of KSt and ASt represent a more flexed joint. The smaller RO represents a narrower range, and the smaller CO represents forward shift of the limb angle. Forward-shifted CO implies less push off at the end of stance phase (Supplementary Fig. 1).

2.5. Muscle mass

Immediately after the motion capture, the animals were euthanized by exsanguination following injection with a lethal dose of sodium pentobarbital (10.37

mg/100 g) [29]. Subsequently, the medial gastrocnemius (MG) and soleus (Sol) were excised bilaterally [25]. The muscles were weighed after trimming off excessive connective tissue, and the muscle weights were normalized according to the whole body weight of each rat (relative weight).

2.6. Extent of recovery from unloading

To determine the extent of recovery after 2 weeks (immediately after the termination of unloading) to 4 weeks (at the end of the recovery period), the percent of recovery (% recovery) of muscle mass and kinematics properties was calculated. The % recovery was determined according to modified equation borrowed from studies by D'Aunno et al. [1,24].

$$\% Recovery = \frac{(UL\ group\ at\ 4\ weeks) - (UL\ group\ at\ 2\ weeks)}{(Ctrl\ group\ at\ 2\ weeks) - (UL\ group\ at\ 2\ weeks)} \times 100$$

This enabled the comparison of the extent of recovery in specific units.

2.7. Statistics

For the data pertaining to legs, values of the right side were included for analysis. All data are expressed as means $\pm$ standard error of the mean (SE). The differences between time points and between groups at each time point were analyzed using two-way analysis of variance (ANOVA) with two factors (group, time point) followed by the Tukey–Kramer post hoc test. Shapiro-Wilk test and Levene's test was selected to confirm the goodness of the normal distribution and equality of variances, respectively. The level of significance was set at $p < 0.05$. Data were analyzed using JMP version 11 (SAS Institute Ltd., Tokyo, Japan).

3. Results

3.1. Body weight

At 2 weeks, the mean whole body weights of the experimental groups (i.e., the UL, UL+1G, and UL+2G groups) were significantly lighter than that of the Control group (Table 1: $p < 0.01$, $p < 0.05$, and $p < 0.01$, respectively). The interaction between two factors (group, time) was significant (Table 1: $p < 0.01$). Within the experimental groups, the UL and the UL+2G groups' weights were significantly lighter than that of the UL+1G group (Table 1: $p < 0.05$ and $p < 0.01$, respectively). At 4 weeks, there was no significant difference between the experimental groups with respect to body weight, although they were still significantly lighter than the Control group (Table 1: $p < 0.01$, $p < 0.05$, and $p < 0.01$, respectively).

3.2. Muscle masses

For the muscle weights, the interaction between group and time was significant (Fig. 1: $p < 0.01$). At 2 weeks, all experimental groups exhibited significantly lower relative weight (% weight) in MG and Sol than Control group (Fig. 1A, C: $p < 0.01$). At 4 weeks, MG had recovered to a weight close to the Control groups' (Fig. 1B: $p > 0.05$). Sol also exhibited recovery close to the Control group although the extent was less than MG (Fig. 1D: $p > 0.05$). No significant difference was found within the experimental groups in either muscle, at either time point. Similar changes were found in absolute muscle weights (before normalized by whole-body weight: Supplementary Fig. 3) although the recovery in Sol was less compared to those in MG.

3.3. Kinematics properties

On the first day after unloading (the first day of the recovery period), in contrast to the Control group, rats in the experimental groups were reluctant to move on the treadmill and they performed shorter durations of locomotion because of fatigue. Nevertheless, they could perform an alternating pattern in their two hindlimbs during walking.

3.3.1. Joint displacements

Fig. 2 illustrates the trajectories of the knee and ankle joint displacements in a step cycle of representative subjects: 0% of a step cycle represents paw contact, and 100% represents the next paw contact of the same limb. In the Control group, double-peak motion (extensions at the ending of the stance and swing phase) and flexion during the initial stance phase were observed in the knee and ankle throughout the experiment (Fig. 2A–C: the arrows and curved striped line, respectively; Supplementary Video 3, 4). However in the UL group at 2 weeks, the preceding peak of the double-peak motion was less pronounced, and flexion during the stance phase was reduced, which is indicative of hyperextension during the stance phase (Fig. 2D: the round tipped bars and the straight double bars, respectively; Supplementary Video 5). Further, those altered motions had not fully returned to the initial state at 4 weeks (Fig. 2E; Supplementary Video 6). Also, in the UL+1G group, the first peak was less pronounced, and hyperextension during the initial stance phase was present at 2 and 4 weeks, although these motion disruptions were slightly less prominent at 4 weeks (Fig. 2F, G: the round tipped bars and the straight double bars, respectively;

Supplementary Videos 7, 8). However, in the UL+2G group, double-peak motions were less affected and hyperextension was decreased by 4 weeks (Fig. 2H, I: the arrows and the curved striped lines, respectively; Supplementary Videos 9, 10).

3.3.2. Knee and ankle angles at stance phase

3.3.2.1. Knee angle

The knee joint trajectory of the UL+2G group was closer to that of the Control group than to those of the other experimental groups both at 2 and 4 weeks (Fig. 3 A, B: black lines). Regarding the mean KSt at 2 weeks, the UL and UL+1G group exhibited significantly greater extension than the Control group (Fig. 3C, 2 weeks: $p < 0.01$ for both). On the other hand, the UL+2G group was not significantly different from the Control group (Fig. 3C, 2 weeks: $p > 0.05$). These differences in the UL and UL+1G groups from the Control group persisted at 4 weeks (Fig. 3C, 4 weeks: $p < 0.01$ for both); in contrast, the UL+2G group was not significantly different from the Control group (Fig. 3C, 4 weeks). The interaction between group and time was significant (Fig. 3C: $p < 0.01$).

3.3.2.2. Ankle angle

The ankle joint trajectory of the UL+2G group was similar to that of the Control group in contrast to those of the other experimental groups both at 2 and 4 weeks (Fig. 4A and B). Regarding the mean ASt, similar changes to those of knee joints were observed (Fig. 4C). The UL and UL+1G group showed significantly greater extension than the Control group at 2 weeks (Fig. 4C, 2 weeks: $p < 0.01$ for both), whereas the

UL+2G group was closer to the Control despite the difference was still significant (Fig. 4C, 2 weeks: $p < 0.01$). The differences of the UL and UL+1G group from the Control group persisted at 4 weeks (Fig. 4C, 4 weeks: $p < 0.01$ for both), while no significant difference was found between the UL+2G group and the Control group (Fig. 4C, 4 weeks). The interaction between group and time was significant (Fig. 4C: $p < 0.01$).

3.3.3. Limb angles

At 2 weeks, RO in all experimental groups was significantly narrower than that in the Control group (Fig. 5C compared to 5A, shaded sections for the outline; Fig. 5E, 2 weeks for the mean value: $p < 0.01$, respectively). However, these differences disappeared at 4 weeks (Fig. 5E, 4 weeks: $p > 0.05$). The interaction between group and time for RO was not significant (Fig 5E: $p > 0.05$). Regarding CO, the UL and UL+1G groups exhibited significant forward shift than the Control group (Fig. 5D compared to B, shaded sections for the outline; Fig. 5F, 2 weeks for the mean value: $p < 0.01$ for both), whereas the UL+2G group was not different from the Control group (Fig. 5F, 2 weeks: $p > 0.05$). The differences in the UL and UL+1G group from the Control group persisted at 4 weeks (Fig. 5F, 4 weeks: $p < 0.01$ for both); meanwhile, the CO in the UL+2G group did not differ significantly from that in the Control group throughout the experimental period (Fig. 5F: $p > 0.05$). The interaction between group and time for CO was significant (Fig 5F: $p < 0.01$).

3.4. Extent of Recovery from Unloading

Percent recovery exhibited different extent between muscle and kinematics properties (Table 2). Regarding muscles, experimental groups exhibited similar recoveries. On the other hand, gait parameters in general exhibited substantially greater recoveries in the UL+2G group compared to the UL and UL+1G groups.

4. Discussion

There are two main findings of the present study as follows: (1) two weeks of simulated microgravity induced locomotor alterations in rats, which did not recover after 2 weeks of reloading despite the recovery in muscle atrophy; (2) exposure to intermittent hypergravity (2G) during the unloading period attenuated the locomotor alterations even though there was little systematic difference in muscle recovery in the rats submitted to 1G reloading or continuous unloading.

4.1. Muscle mass

Muscle mass recovered within 2 weeks after reloading (i.e., at 4 weeks) following the transient decrease due to unloading (i.e., at 2 weeks). Although the extent of recovery varied owing to the distinct responsiveness to weightlessness [30], the muscular adaptations observed are concordant with those of other studies showing unloading-induced atrophy in hindlimb or leg extensor muscles followed by recovery after reloading [31–36].

The percent recoveries of the Sol were less than those of MG. This is reasonable because Sol is more susceptible to weightlessness than other muscles [2]. In terms of

group difference, the recoveries were similar across the experimental groups. This is also consistent with previous studies.

Indeed, D'Aunno et al. show that neither 1.5 nor 2.6G application for 1 hour per day enhances the relative muscle weights in rats during hindlimb unloading [1]. Concordant with their study, other studies show that the application of hypergravity only slightly affects muscle atrophy with respect to muscle weight, although centrifugation could interfere with muscle degradation in terms of alterations in myosin heavy chain [37] or enzymes [38]. Because muscle properties in weightlessness are affected by the form of activity while the subjects are in microgravity environment [39], it can be surmised that light exercise, which is at least more active than stationary ground support, is required to improve the functional aspects of muscles even when hypergravity countermeasures are employed [17].

4.2. Kinematics properties

In the case of intact locomotion, walking is a rhythmic motion with inter-limb coordination (i.e., pendulum-like motion between right and left hindlimbs) and intra-limb coordination (i.e., flexion and extension in the same limb) [7]. Canu et al. demonstrate that 2 weeks of hindlimb unloading disturbs intra-limb coordination, whereas inter-limb coordination is less affected [10]. Similar alterations were observed in the present study. Rats in the UL group walked with their hindlimb overextended during stance phase after 2 weeks of hindlimb unloading. To our knowledge, our study is the first to show that the double-peak motions of hindlimb joints, which are

observed in intact locomotion [27], are disturbed by microgravity environments. Furthermore, modifications to the motion did not simply revert to the original state after 2 weeks of recovery [9]. This is consistent with the study by Canu et al., which allows the possibility of the persistence of altered locomotion after reloading [8]. The sustained motion alterations are also concordant with those of another study showing that 2 weeks of hindlimb unloading results in a long-lasting alterations in neurogenesis, which are not restored merely because the perturbation is removed, despite exercise as a countermeasure [40]. In order to quantify these gait alterations, in the present study, joint parameters were evaluated. The results showed significant extensions in the KSt and ASt, a narrower RO, and a forward-shifted CO immediately after the termination of unloading in the UL group. Furthermore, except for the RO, neither 2 weeks of reloading for the recovery period nor adding intermittent 1G application during the unloading period resulted in these parameters being fully restored after the termination of unloading.

Microgravity environments evoke alterations in proprioceptive information [41,42], which could consequently modify motor output [43]. Other studies concordantly show that microgravity alters neural structures that innervate hindlimbs, such as the motor cortex [44,45], dendritic spines [46], the spinal cord [42], succinate dehydrogenase activity [41], and GABAergic cells in the somatosensory cortex [47,48]. Although the present study did not evaluate the ascending inputs, it could be surmised that microgravity enhances the responsiveness of somatosensory neurons by down-regulated GABAergic function [47,48] and reduced threshold [49]. When subjects are subsequently returned to normal gravity and the demand for

weight bearing consequently increases on decreased motor cortex [44,45], hindlimb joints might be excessively activated [42]. These alterations of the central nervous system could account for the observed deficits in gait. Further, Yasuhara et al. referred to the perseverance of the neural alteration due to the microgravity. They suggested that neurogenesis in rats' hippocampus is inhibited by hindlimb unloading, and still suppressed after the termination of the unloading [40].

On the other hand, in the present study, the rats in the UL+2G group exhibited significantly smaller deficits in joint trajectories and parameters than both the UL and UL+1G groups, despite the similarity in muscle recovery to those groups. Although the precise reason for this discrepancy between muscle and kinematics properties is unknown, the alterations of locomotion after exposure to hypergravity have been previously confirmed. Bouet et al. studied rats' locomotion exposed to hypergravity. Although their comparison was between hypergravity and cage control without unloading, the animals in their study walked "more flexed and closer to the ground" after exposure to hypergravity [14]. They express these observations as being opposite those due to microgravity. They subsequently infer that increased input of proprioceptive information due to hypergravity desensitized the central nervous system [14]. Indeed, as locomotion is modulated by the convergence of descending command and ascending information [50,51], alterations in proprioceptive information could modify motor output [43]. Other studies also support this hypothesis [52–54]. Regarding alterations of the central nervous system, several studies investigating the effects of hypergravity indicate the existence of vestibular adaptations [55,56] including structural alterations in the lateral vestibular nuclei [57], a decreased

number of macular hair cells [58], and a decrease in the relative size of the utricle otoconia [59]. Furthermore, Borisova et al. demonstrate that centrifuge-induced hypergravity evokes enhancement of GABA (i.e., inhibitory) and reduction of glutamate (i.e., anti-excitatory) neurotransmitter release in the cerebral hemisphere in rats [60].

To the extent of our knowledge, none of these aforementioned studies subjected rats to centrifugation while they were exposed to microgravity environments. On the other hand, the present study indicates that multiple modifications due to hypergravity could inhibit gait alterations induced by simulated microgravity. Therefore, it could be assumed that intermittent application of hypergravity using a centrifuge might counteract the alterations in gait induced by microgravity environments.

Despite its strengths, this study has some limitations. First, although UL+2G application maintained locomotion closer to those of intact individuals, this was merely based on behavioral observations. Therefore, it is still difficult to conclude whether the present results are attributable to the resolution of the adverse effects of microgravity or to another adverse effect of hypergravity. To address this issue, other walking performance tests as well as joint displacements must be performed. Furthermore, if there are disadvantages to hypergravity application, a break-even point in the duration or intensity of the intervention should be identified. Second, to more precisely understand the changes in muscles, properties such as the transitions of muscle fiber type (slow to fast or vice versa) or myosin heavy chain mRNA expression should have been examined. Moreover, although muscles are less likely to

influence the observed disturbances in locomotion, the most accountable factor still remains unknown. Studies focusing on neural structures are required to clarify this.

4.3. Conclusions

In summary, intermittent application of hypergravity by centrifugation may counteract gait alterations in rats induced by simulated microgravity environments. These findings imply the existence of responsible factors, such as modifications of neural structures, other than the recovery of hindlimb muscle atrophy. However, the mechanism in detail as well as optimal duration and intensity must be more precisely identified in order to take advantage of hypergravity as a countermeasure against microgravity.

Acknowledgements

This study was supported in part by JSPS KAKENHI Grants-in-Aid for Scientific Research (A) (#25242055) and Challenging Exploratory Research (#25560258).

Authors would like to thank Bungo Otsuki (Department of Orthopaedic Surgery, Kyoto University Hospital, Kyoto, Japan) for fundamental concept of the centrifuge for small animals. Also, thank Takahiro Fukumoto (Faculty of Health Science, Kio University, Nara, Japan) for the contribution in development of the device.

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Fig. 1

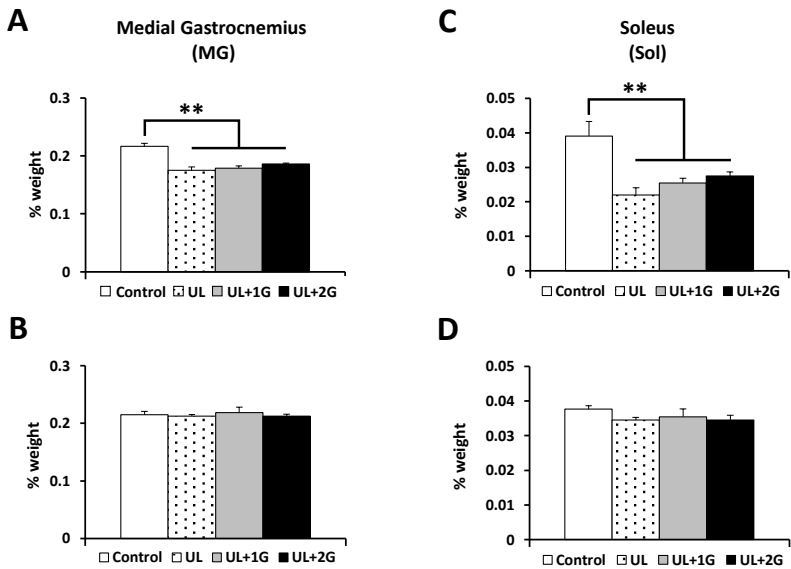


Fig. 2

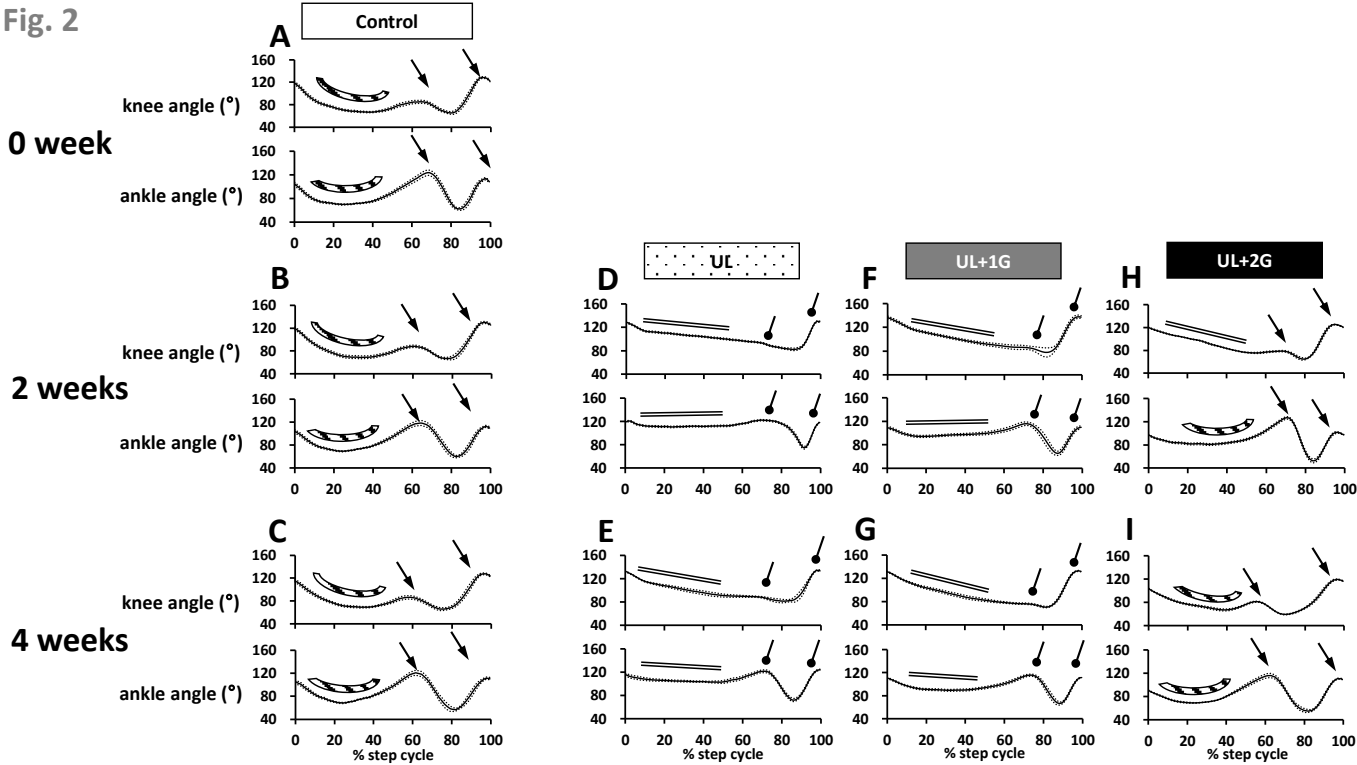


Fig. 3

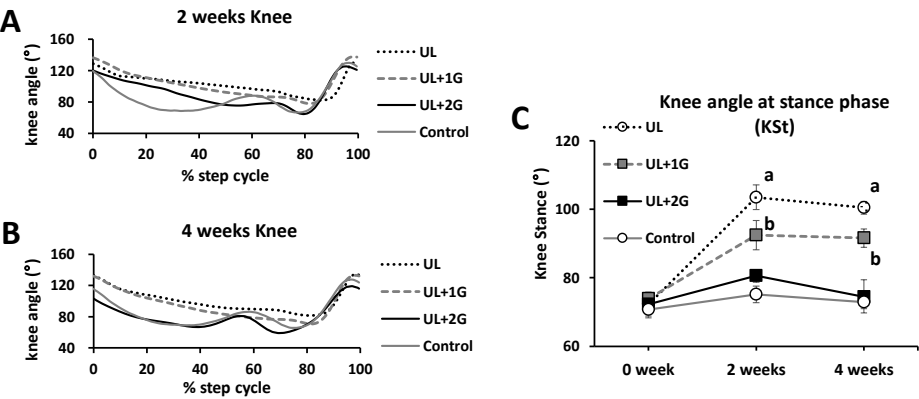


Fig. 4

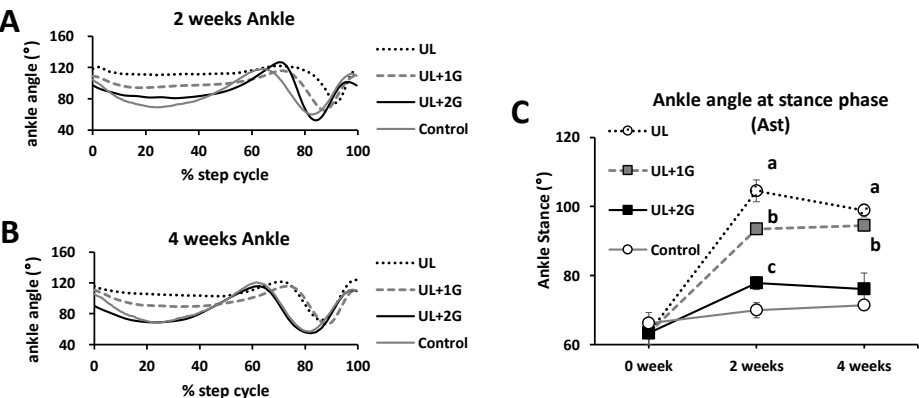
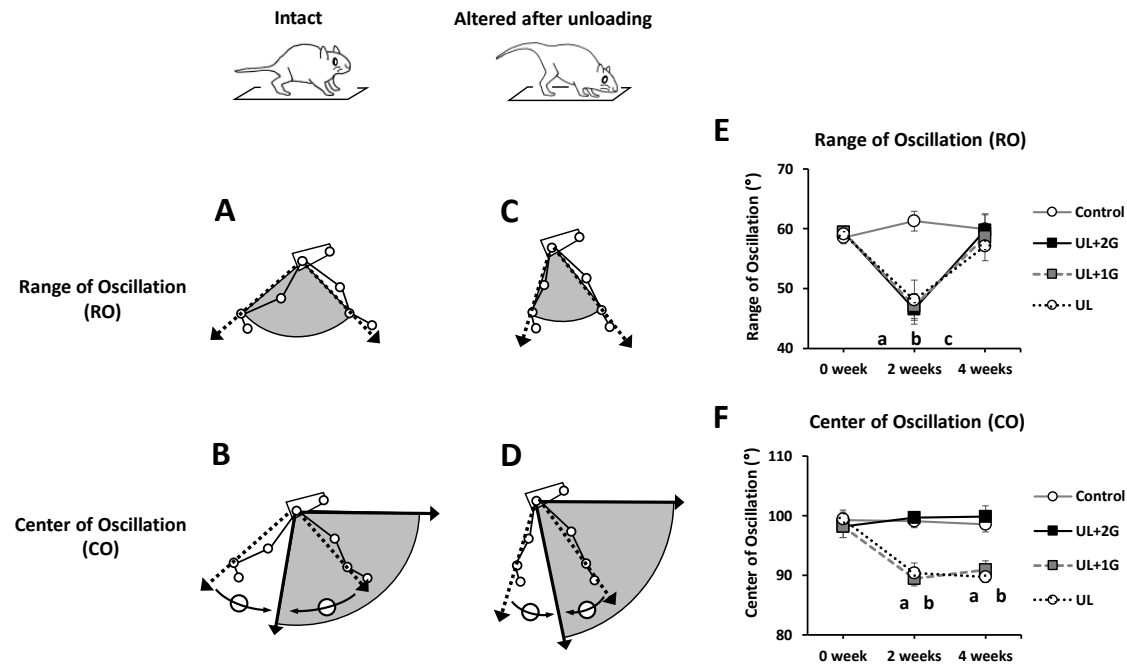
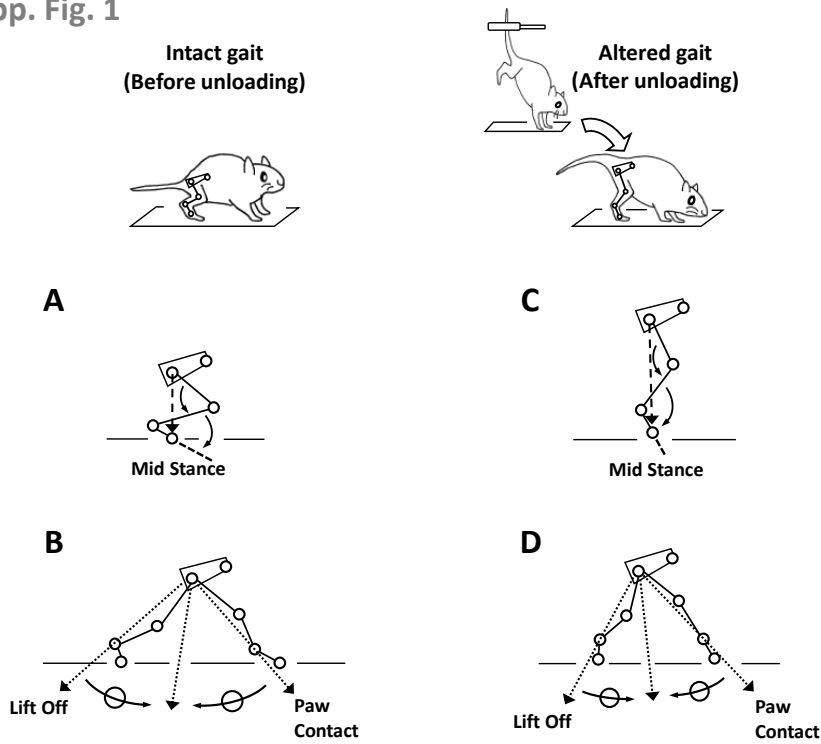


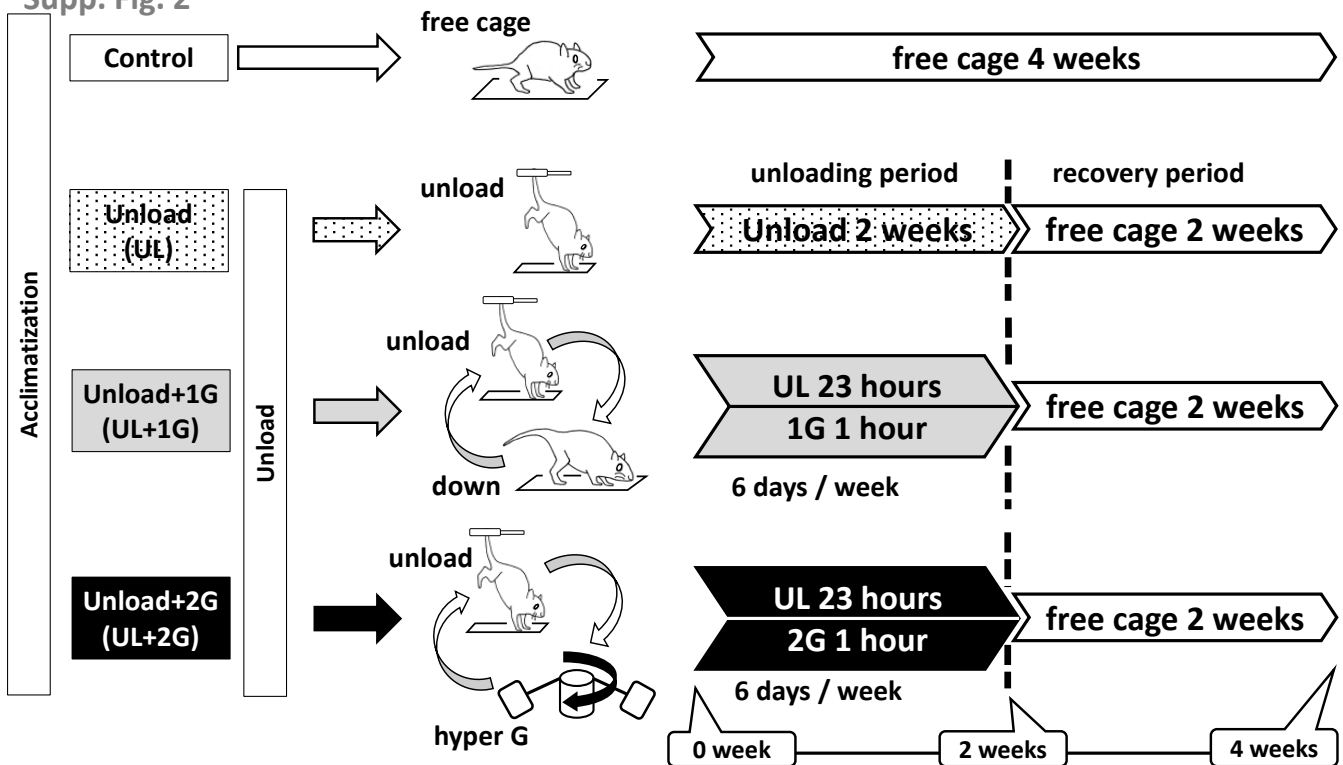
Fig. 5



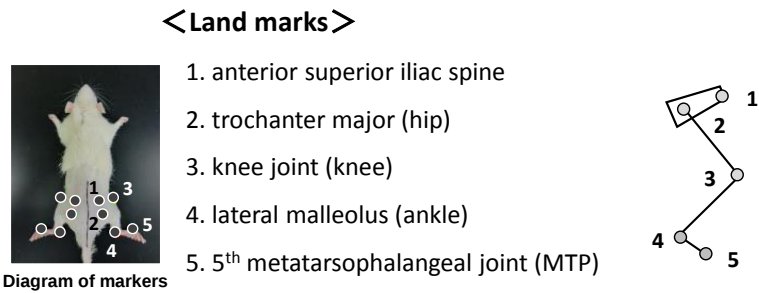
Supp. Fig. 1



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Supp. Fig. 4

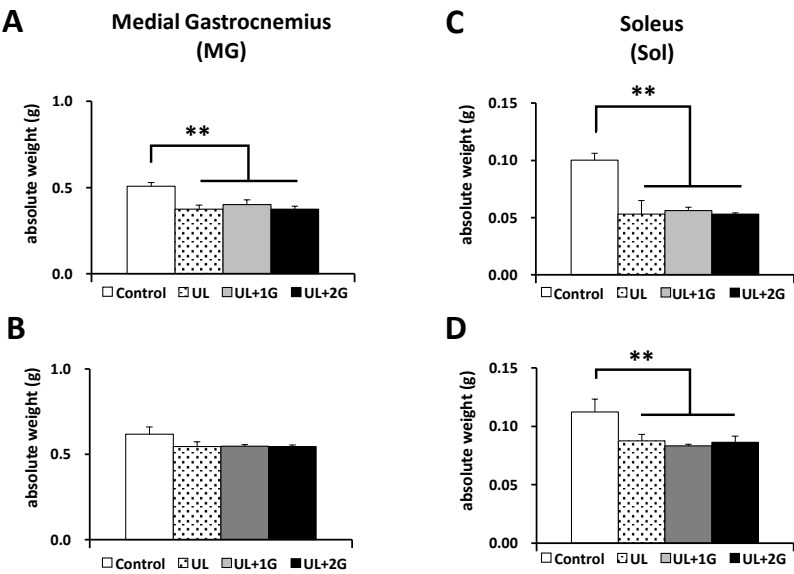


FIGURE LEGENDS

Fig. 1 Change of the muscle relative weight to the body weight.

At 2 weeks, all experimental groups (i.e., the UL, UL+1G, and UL+2G) displayed a significant decrease from the Control group in both MG (A) and Sol (C). At 4 weeks, MG recovered to a level close to the Control group (B). Sol also exhibited recovery close to the Control although the extent was less compared to MG (D). No significant difference was found among the experimental groups throughout the time line.

** $p < 0.01$ to Control

(two-way ANOVA, interaction: $p < 0.01$ followed by Tukey post hoc)

Fig. 2 Trajectories of joint excursions of representative subjects.

0 week (before the unloading: A), 2 weeks (immediately after the termination of the unloading: B, D, F, H), 4 weeks (2 weeks after the reloading: C, E, G, I) of a step cycle from representative subjects. 0 % of step cycle is the paw contact. 100 % is the next paw contact of the same limb. Dotted line represents $\pm$ standard error of the mean. In the Control group at 0 week (before unloading), double-peak motions (extensions at the ending of the stance and swing phase) and flexion during the initial stance phase were observed in the knee and ankle (A, B, C: arrows and curved striped lines, respectively). However in the UL group at 2 weeks, the double-peak motions (the

first peak of the double-peak motions) were less pronounced and flexion during the initial stance phase was reduced, which represent hyperextension of the hindlimb (D: round tipped bars, straight double bars, respectively). Those altered motion persisted at 4 weeks (E). The UL+1G group also showed the similar changes (F, G). However, in the UL+2G group, double peak motions were less affected and hyperextensions were decreased by 4 weeks (H, I: arrows and round tipped bars, respectively).

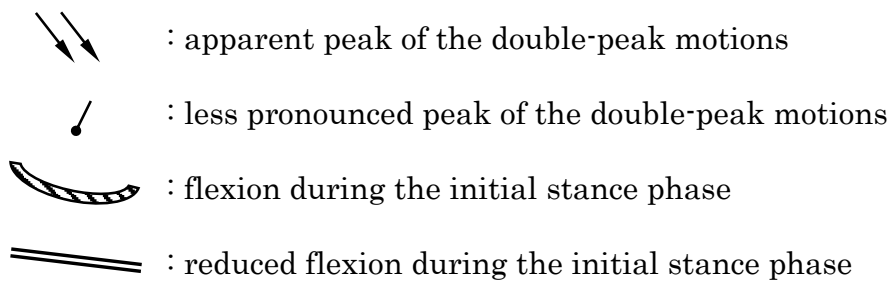


Fig. 3 Overlapped graphs of knee joint trajectories of representative subjects from each group and the mean KSt (knee angle when hip is vertical with MTP) in each time point of interest.

The joint trajectory of the UL+2G group was closer to those of the Control group than other experimental groups both at 2 weeks and 4 weeks (4A, B: black lines). For the KSt, the UL and UL+1G group showed significantly greater extension than the Control group at 2 weeks (C: 2 weeks, $p < 0.01$ for both). On the other hand, the UL+2G group was not significantly different from the Control group (C: 2 weeks, $p > 0.05$). Those difference of the UL and UL+1G groups from the Control group persisted at 4 weeks, while the UL+2G group was not significantly different from the

Control group (C: 4 weeks, $p < 0.01$ for the UL and UL+1G group, $p > 0.05$ for the UL+2G group).

a UL group $p < 0.01$ to Control

b UL+1G group $p < 0.01$ to Control

(two-way ANOVA, interaction: $p < 0.01$ followed by Tukey post hoc)

Fig. 4 Overlapped graphs from the representative subjects and the mean KSt (ankle angle when hip is vertical with MTP) in the identical configuration of Fig. 3.

The joint trajectory of the UL+2G group was similar to those of the Control group in contrast to other experimental groups both at 2 weeks and 4 weeks (A, B). For ASt, the UL and UL+1G group showed significantly greater extension than the Control group at 2 weeks (C: 2 weeks, $p < 0.01$ for both). On the other hand, the UL+2G group kept closer to the Control group although the difference between them was significant (C: 2 weeks, $p < 0.01$). Those differences of the UL and UL+1G from the Control group persisted at 4 weeks, in contrast to the UL+2G group that was no longer different from the Control group (C: 4 weeks, $p < 0.01$ for the UL and UL+1G group, $p > 0.05$ for the UL+2G group).

a UL group $p < 0.01$ to Control

b UL+1G group $p < 0.01$ to Control

c UL+2G group $p < 0.01$ to Control

(two-way ANOVA, interaction: $p < 0.01$ followed by Tukey post hoc)

Fig. 5 Transitions of limb angles.

Outline pictures for RO and CO (shaded sectors in A, C and B, D, respectively). Mean angles at each time point of interest in RO (E) and CO (F). Panel A and B outline intact motions, and panel C and D for altered motions (not specific for the groups). On E and F, smaller degree means narrower oscillation for RO, and forward-shifted limb motion for CO respectively. At 2 weeks, RO of all three experimental groups were significantly narrower (smaller) than the Control group (E: 2 weeks, $p < 0.01$ for each). Those differences disappeared over time (E: 4 weeks, $p > 0.05$ for each). As for the CO, the UL and UL+1G group showed forward-shift (smaller degrees) than the Control group at 2 weeks, whereas the UL+2G group did not show difference (F: 2 weeks, $p < 0.01$ for the UL and UL+1G group, $p > 0.05$ for the UL+2G group, respectively). Those differences of the UL and UL+1G group from the Control group persisted at 4 weeks, while the UL+2G group did not show significant difference from the Control group throughout the experimental period (F: 4 weeks, $p < 0.01$ for the UL and UL+1G group, $p > 0.05$ for the UL+2G group, respectively).

- a** UL group $p < 0.01$ to Control
- b** UL+1G group $p < 0.01$ to Control
- c** UL+2G group $p < 0.01$ to Control

(two-way ANOVA, interaction: $p > 0.05$ for RO, and $p < 0.01$ for CO followed by Tukey post hoc)

Supplementary Figure 1. Outline of the rat's locomotion that is intact or altered by microgravity environment.

When rats are exposed to microgravity environment and are back to the normal gravity situation subsequently, their knee and ankle is overextended during stance phase (C) compared to the intact gait (A). The altered locomotion also embraces narrower and forward-shifted oscillation of the hindlimb (D) compared to the intact gait (B).

Supplementary Figure 2. Detail of the experimental time course.

After the acclimatization to the treadmill, the rats in the UL, UL+1G, UL+2G group were unloaded for the initial 2 weeks (unloading period). During the unloading period, the rats in the UL group were kept unloaded throughout the period. For the UL+1G group, animals were relieved down on the ground for 1 hour a day, 6 days a week. For the UL+2G group, animals were submitted to the hypergravity by means of centrifugation in the identical timing of the UL+1G group. After the unloading period, they were re-loaded and kept freely for another 2 weeks (recovery period) until the final evaluation. The evaluations were carried out in every 2 weeks. Six animals were extracted at each time point of interest (0 week [beginning of the unloading period], 2 weeks [termination of the unloading period], 4 weeks [ending of the recovery period]) and subjected to the subsequent data collection.

Supplementary Figure 3. Diagrams of rat's land marks and plastic markers, which corresponded to those landmarks.

Supplementary Figure 4. Absolute weights of muscles.

In general, no significant difference was observed among the three experimental groups (A: MG at 2 weeks; B: MG at 4 weeks; C: Sol at 2 weeks; D: Sol at 4 weeks) although they were significantly lighter to the Control group except for the MG at 4 weeks (B).

****** $p < 0.01$ to Control

(two-way ANOVA, interaction: $p < 0.01$ followed by Tukey post hoc)

Table 1. Changes of the whole body weight.

Groups	Time points		
	0 week	2 weeks	4 weeks
Control	203.4±4.1	235.4±8.3	289.9±9.9
UL	202.7±3.8	212.9±5.3 b,c	257.1±7.4 b
UL+1G	200.3±4.1	226.8±9.2 a	265.1±4.3 a
UL+2G	200.8±3.0	203.0±7.2 b,d	260.4±6.0 b

At 2 weeks, body weights of experimental groups were significantly smaller than those in the Control group. Further, the UL and UL+2G were significantly lighter than UL+1G. At 4 weeks, the differences among the experimental groups disappeared although they were still significantly lighter than the Control group.

a $p < 0.05$ to Control, **b** $p < 0.01$ to Control

c $p < 0.05$ to +1G, **d** $p < 0.01$ to +1G

(two-way ANOVA, interaction: $p < 0.01$ followed by Tukey post hoc)

Table 2. The percent recovery of muscle mass and kinematics parameters.

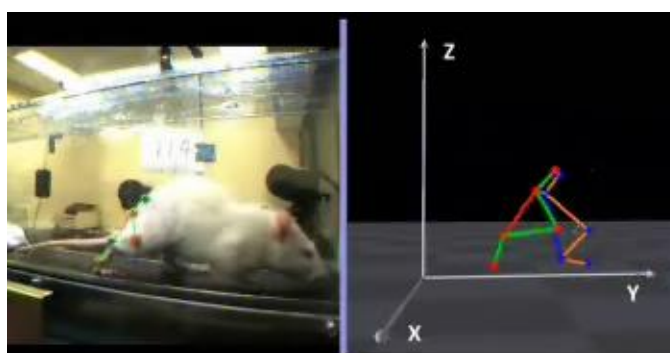
Groups	Muscles		Gait parameters			
	MG	Sol	KSt	ASt	RO	CO
UL	90.6	73.0	31.8	12.1	74.5	21.2
UL+1G	100.4	78.8	52.1	21.3	85.4	49.3
UL+2G	90.6	73.4	89.6	91.3	106.5	106.7

Muscle mass exhibited similar recoveries across the groups. On the other hand, gait parameters exhibited greater recoveries in the UL+2G compared to that in the UL and UL+1G groups.

Supp. Video 1



Supp. Video 2



Supp. Video 3

**Ctrl
0 wk**



Supp. Video 4

**Ctrl
4 wk**



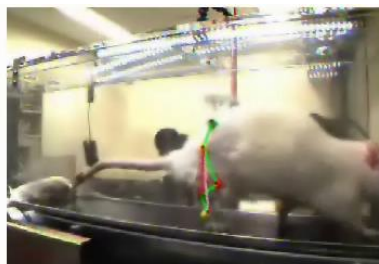
Supp. Video 5

**UL
2 wk**



Supp. Video 6

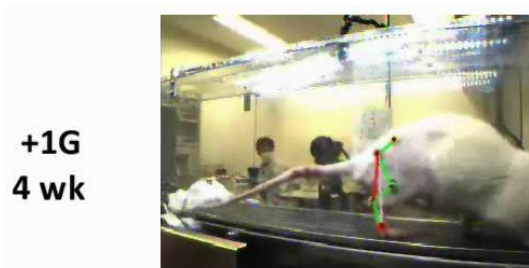
**UL
4 wk**



Supp. Video 7



Supp. Video 8



Supp. Video 9



Supp. Video 10



VIDEO LEGENDS

Supplementary Video 1. Centrifuge for small animals.

Animals in the UL+2G group were subjected to hypergravity (2G) for 1 hour per day, 6 times per week.

Supplementary Video 2. Rat's walking and 3-dimensional movements reconstructed from the captured movies.

Note the 3-D directions for the x-, y-, z-axes were lateral, anterior and vertical, respectively (i.e., right-hand rule).

Supplementary Video 3. Walking of Control group at 0 week.

Supplementary Video 4. Walking of Control group at 4 weeks.

Supplementary Video 5. Walking of UL group at 2 weeks.

Knee and ankle joints are hyperextended, and hindlimbs are more protracted.

Supplementary Video 6. Walking of UL group at 4 weeks.

The disruption in limb motion are not fully reverted to the previous state.

Supplementary Video 7. Walking of UL+1G at 2 weeks.

Similar to those of the UL group, knee and ankle joints are hyperextended, and hindlimb are more protracted.

Supplementary Video 8. Walking of UL+1G at 4 weeks.

Intermittent 1G application did not restore the gait alteration at 4 week.

Supplementary Video 9. Walking of UL+2G at 2 weeks.

Knee and ankle joints are slightly flexed during the stance phase, and the limb protraction is less pronounced compared to other experimental groups.

Supplementary Video 10. Walking of UL+2G at 4 weeks.

Their gait were closer to those of the Control group compared to other experimental groups.