Mild-intensity running exercise recovered motor function by improvement of ankle mobility after unilateral brain injury of mice using three-dimensional kinematic analysis techniques

Akira Yoshikawa a,b, Hirokazu Ohtaki c,d,e, Kazuyuki Miyamoto f, SungHyek Kim g, Kazunori Hase h, Makoto Yoshida i, Shotaro Kamijo a,h, Sawa Kamimura a,i, Nobuyoshi Koiwa j, Masahiko Izumizaki a

a Department of Physiology, Showa University School of Medicine, 1-5-8 Hatano-dai, Shinagawa-ku, Tokyo 142-8555, Japan
b Division of Health Science Education, Showa University School of Nursing and Rehabilitation Sciences, 1865 Tokaiichibaye, Midori-ku, Yokohama 226-0025, Japan
c Department of Anatomy, Showa University School of Medicine, 1-5-8 Hatano-dai, Shinagawa-ku, Tokyo 142-8555, Japan
d Department of Functional Neurobiology, Tokyo University of Pharmacy and Life Sciences School of Pharmacy, 1432-1 Hiratsuka, Hachiouji, Tokyo 192-0392, Japan
e Department of Emergency, Critical Care and Disaster Medicine, Showa University School of Medicine, 1-5-8 Hatano-dai, Shinagawa-ku, Tokyo 142-8555, Japan
f Department of Shizuoka Physical Therapy, Tokoha University Faculty of Health Science, 1-30 Mizuochicho, Aoi-ku, Shizuoka 420-0831, Japan
g Department of Mechanical Systems Engineering, Tokyo Metropolitan University Faculty of Systems Design, 1-1 Minami-Osawa, Hachiouji-shi, Tokyo 192-0397, Japan
h Department of Physiology, Showa University School of Pharmacy, 1-5-8 Hatano-dai, Shinagawa-ku, Tokyo 142-8555, Japan
i Department of Otorhinolaryngology Head and Neck Surgery, Showa University School of Medicine, Tokyo, Japan, 1-5-8 Hatano-dai, Shinagawa-ku, Tokyo 142-8555, Japan
j Department of Anatomy, Showa University School of Medicine, 1-5-8 Hatano-dai, Shinagawa-ku, Tokyo 142-8555, Japan

ABSTRACT

Motor dysfunction, such as gait impairment, is a major disability induced by traumatic brain injury or stroke. Treadmill running is often used as a physical exercise (Ex) clinically and experimentally for the recovery of patients. In animal experiments, although dynamic behavioral deficits can be evaluated using scoring systems, local and minor behaviors are difficult to determine. This study aims to evaluate motor dysfunction and recovery after brain damage (BD) with/without mild-intensity running Ex in mice using three-dimensional (3D) kinematic analysis. To determine exercise intensity, C57/BL6-strain male young adult mice were examined in an incremental running test while the pulmonary gas exchange of O₂ and CO₂ were measured. The animals were then subjected to left hemidecortication as BD, and some mice performed Ex (10 m/min for 30 min 5 times/wk) for 4 weeks. The BD with Ex and BD or sham-operated mice (sham) without (w/o) Ex had their gait recorded by four synchronized cameras, and gait was evaluated via 3D-kinematic analysis. The BD w/o Ex mice significantly differed in stride, step, and stride width for both limbs compared to the sham w/o Ex mice. The BD with Ex mice showed improvement. The BD w/o Ex mice had restricted ankle movements and impairment in dorsal/plantar flexing using trajectory analysis. Consistent with these impairments, the nonaffected side also exhibited a different trajectory, suggesting compensatory movements. These results suggest that the appropriate Ex after BD recovered motor function. Furthermore, the present study suggested that 3D-kinematic analysis is a powerful tool for detecting minor behavioral alterations owing to the impairment of the affected side and the compensation of the unaffected side.

1. Introduction

Motor dysfunction such as gait impairment is a major disability after a traumatic brain injury or stroke, and results in the deterioration of activities of daily living (ADL) and/or quality of life (QOL) (Belda-Lois et al., 2011; Jorgensen et al., 1995; Olney and Richards, 1996). Rehabilitation therapy to regain partially or completely deprived functions is important to improve the ADL/QOL (Belda-Lois et al., 2011; Buurke...
et al., 2008; Mauritz, 2002). While there are many therapeutic approaches, such as physical, occupational, and speech-language therapy, physical therapy is the most important approach for gait impairment (Kwakkel et al., 2004). However, the mechanism by which exercise improves motor dysfunction is still unclear. To elucidate this mechanism of recovery, the animal model is one of the important tools of research (Murphy and Corbett, 2009; Nudo et al., 1996).

To support the recovery of physical activities after brain damage (BD) in animals, treadmill running is often used as a physical exercise task (Endres et al., 2003; Morishita et al., 2020; Taguchi et al., 2019; Wang et al., 2020). Following this task, the motor functions of rodents were examined using various tests, such as the reach and grasp task, staircase pellet reaching test, tape removal test, ladder/beam walking test, and the rotarod test (Schaar et al., 2010; Starkey et al., 2005). These behavioral examinations are scored by the degree of attainment and/or time on the task. These scorings can be evaluated as primary screening approaches, such as physical, occupational, and speech-language therapy, physical therapy is the most important approach for gait impairment (Kwakkel et al., 2004). However, the mechanism by which exercise improves motor dysfunction is still unclear. To elucidate this mechanism of recovery, the animal model is one of the important tools of research (Murphy and Corbett, 2009; Nudo et al., 1996).

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2. Results

A schematic illustration of the experimental design is shown in Fig. 1. Briefly, we assessed the physical fitness of mice before subjecting them to either left hemidecortication as brain damage (BD) or the sham-operation (Fig. 1A and B). Beginning one day after the surgery and continuing for four weeks, BD with treadmill running exercise (Ex) mice performed 30 min of treadmill running five times/week (Fig. 1B). Mice in the BD without Ex (BD w/o Ex) group and sham w/o Ex groups were

Fig. 1. Schematic overview of this experiment. A, The mice were divided into three groups as follows: brain damage (BD) with treadmill running exercise (Ex) (BD with Ex, n = 10 males), BD without Ex (BD w/o Ex, n = 8 males), sham-operation without Ex (sham w/o Ex, n = 8 males). Physical fitness of the BD with Ex mice was assessed by the incremental running test before left hemidecortication (as a BD). All group’s mice underwent the operation (day 0). The running exercise was initiated the day after the operation and continued for 4 weeks for the BD with Ex mice. Monitoring of VO₂ and VCO₂ in BD with Ex animals was conducted 4 times at 8 (1 week), 15 (2 weeks), 22 (3 weeks), and 29 (4 weeks) days after the left hemidecortication. In all groups, the gait and ankle joint were measured at 30 or 31 days (4 weeks) after the left hemidecortication by using three-dimensional motion analysis. To evaluate the left hemidecortication, the corticospinal tract was immunostained with PKCγ antibody after recording the gait and ankle joint. B, The treadmill chamber for incremental running test and running exercise. This treadmill chamber was airtight so that air was pushed continuously at 1.0 L/min by ventilation, and the O₂ consumption (VO₂, ml/min/kg) and CO₂ production (VCO₂, ml/min/kg) were measured during running. The RER was calculated by dividing VCO₂ by VO₂. The relationship between treadmill speed (m/min) and incremental running test time (min). C, The points of each joint bonded to the 14 total color beads. Each joint is as follows: the shoulders, elbows, wrists, hips, knees, ankles, and base of little fingers (5th finger) in four limbs. D, The environment of the motion capture system® and stick figures of mice were reconstructed. The right side showed the joints with red marker and the left side showed the joints with blue marker.
maintained in normal animal cages for four weeks. One month after the BD or sham-operation, the gait and movement of the mice were evaluated by 3D-based motion capture technology (Fig. 1C–E).

2.1. Determination of the appropriate intensity of running exercise in healthy mice

The appropriate volume of physical aerobic running exercise in healthy mice (n = 10) was determined by an incremental running test on the treadmill before injury (Figs. 1A, B and 2A). All mice were able to run on the treadmill at a speed up to 44.0 m/min. However, they began dropping out at running speeds of ≥46.0 m/min. No mice were able to run at 54.0 m/min (Fig. 2B). The mean of the maximum running speed was 47.4 ± 2.6 ml/min/kg. The resting VO$_2$ and VCO$_2$ were 78.9 ± 2.7 and 63.1 ± 2.6 ml/min/kg, respectively, and gradually increased during treadmill running. The respiratory exchange ratio (RER), which is the ratio of VCO$_2$ and VO$_2$, also increased with running. When the treadmill speed reached at 30 m/min and the RER was >0.9, the slope became slightly narrower (Fig. 2C). We then calculated the inflection point of aerobic/anaerobic exercise from VO$_2$ and VCO$_2$ (Fig. 2D), and determined them as 108.5 ± 4.1 and 91.5 ± 6.1 ml/min/kg, respectively. This inflection point was indicated at treadmill speeds between 15 and 20 m/min (Fig. 2C square inset). From this experiment, we utilized a treadmill speed of 10 m/min as mild aerobic exercise in the following study.

2.2. Evaluation of mild-intensity running exercise in BD with Ex mice

The VO$_2$, VCO$_2$, and RER of the mice were measured during exercise every week. Before the BD, VO$_2$, VCO$_2$, and RER were 102.6 ± 2.6 ml/min/kg, 83.0 ± 3.0 ml/min/kg, and 0.81 ± 0.01, respectively. After the BD, the VO$_2$, VCO$_2$, and RER ranged from 103 to 106 ml/min/kg, 81 to 86 ml/min/kg, and 0.79 to 0.84, respectively. These values were insignificantly different from those of the healthy mice (Fig. 2E), suggesting that the BD with Ex mice were able to perform mild-intensity running exercise as rehabilitative therapy.

2.3. Kinematic analysis of gait parameters

The sham w/o Ex mice stepped on the treadmill with a rhythmic and fluid motion (Movies 1A and 1B). In contrast, the BD w/o Ex stepped with a random pattern, at times skipping and stopping on the treadmill (Movies 1C and 1D). However, the BD with Ex mice relatively maintained running with 5th finger marker beads (Fig. 3A). There were no significant differences in the times of the one-step cycle and stance phase in either hindlimb among the groups (Fig. 3B and C). However, the time of the swing phase in the BD w/o Ex mice was shorter than that in the other groups in both limbs, and it was significantly different in the left limb (unaffected side) (Fig. 3D). Double-stance phase was not significantly different from those of the healthy mice (Fig. 2E), suggesting that the BD with Ex mice were able to perform mild-intensity running exercise as rehabilitative therapy.

Fig. 2. The measurement of oxygen (O$_2$) consumption and carbon monoxide (CO$_2$) production in the incremental running test and running exercise. A, Relationship between treadmill speed and running time during the incremental running test. The increasing speed of treadmill rotation was 1, 3, 5, 10, 15, and 20 m per min for the first 6 min. After reaching a speed of 20 m/min, the treadmill rotation further increased the speed at 2 m/min. It was possible to carry out a physical fitness assessment in about 20 min by this method. B, Relationship between exercise tolerance and number of mice in the incremental running test. All mice were able to run on the treadmill at a speed up to 44.0 m/min. However, they began dropping out from the experiment at running speeds of 46.0 ml/min and higher. C, Transition of VO$_2$, VCO$_2$, and RER in the incremental running test. The three parameters increased gradually during running. However, the VO$_2$ and VCO$_2$ became closer together and the RER became less inclined when the treadmill speed reached approximately 30 m/min. D, Determination of the inflection point from VO$_2$ and VCO$_2$. From the plot of VO$_2$ and VCO$_2$ (gray open circle), the inflection point (arrowhead) was determined using piecewise regression and two-segmented linear models. The two dashed lines show two types of general linear curves estimated from the open circles, and the solid line is the regression line for indicating the inflection point. Based on these results, the treadmill speed corresponding to the inflection point was indicated by a square in Fig. 2C, E. The results of VO$_2$, VCO$_2$, and RER at pre-operation (incremental running test) and at 1, 2, 3, and 4 weeks after the left hemidecortication at 10 m/min on the treadmill speed. The results of VO$_2$, VCO$_2$, and RER are shown as mean ± SEM. VO$_2$, VCO$_2$ and RER at each week were analyzed by one-way ANOVA followed by Bonferroni’s correction (*p < 0.05, **p < 0.01).
different in either of the hindlimbs among the groups (Fig. 3E).

Next, we determined the stride length, step length, and stride width (cm) of the mice (Fig. 3F). The stride and step of the right limbs (affected side) in BD w/o Ex mice were significantly shorter than those in the sham w/o Ex group (Fig. 3G–H). Moreover, the stride width in the BD w/o Ex mice was significantly greater than that among the other groups (Fig. 3I). The distances from the center of the hip to both ankles and the 5th finger were examined to estimate the distortion in posture (Fig. 3J).

These distances were similar in both limbs of the sham w/o Ex mice (black bar in Fig. 3K, L). However, the distances to the right 5th finger or ankle in both BD groups tended to be shorter than those in sham w/o Ex mice. Moreover, the distances in both left limbs in BD w/o Ex mice were significantly longer than those in either the BD with Ex or sham w/o Ex mice (Fig. 3K, L). These results suggested that the longer stride width in BD w/o Ex mice might be due to outward grounding in the left hindlimb (unaffected side). Indeed, these results also indicated that BD w/o Ex mice had improved posture and steps following mild-intensity running exercise as rehabilitation therapy.

2.4. Trajectory of the ankle by 3D analysis

To evaluate the limb movements, the trajectories of the ankle marker beads in both limbs were determined among the experimental groups (Fig. 4).

On the horizontal plane (x-axis and movement of lateral/medial directions; Fig. 4A), both ankles in the sham w/o Ex group were approximately 1.0 cm from the hip center during standing, and the trajectories were turned outside at the swing phase. The right ankles in both BD groups moved in a slight lateral/medial direction, the trajectory in the BD with Ex resembled that of the sham w/o Ex group (Fig. 4A, right panel). The left ankles in sham w/o Ex mice turned a lateral position at the swing phase and switched quickly to medial at the stance phase; all three groups of mice
had similar trajectory shapes. However, the BD w/o Ex mice exhibited a significantly smaller inward trajectory during the stance phase compared with that of the sham w/o Ex mice. The trajectory in BD w/o Ex animals was indicated the impairment but BD with Ex animals was improved slightly (Fig. 4A, left panel).

On the sagittal plane (y-axis and movement of rostral/caudal directions; Fig. 4B), the trajectories of the right ankles in all groups showed caudal movement during the stance phase and switched to rostral in the swing phases (right panel), while the left limbs showed the opposite pattern (left panel). The trajectory in the BD w/o Ex mice was

Fig. 4. The coordinate analysis for the trajectory of ankle during one-step cycle. A: Illustration of the one-step cycle in horizontal view images and the trajectories of the ankle joints. The trajectory in the horizontal (x-axis and movement of lateral/medial directions) was calculated using the center of the hip as the zero (0) point. B: Illustration of the one-step cycle in the sagittal view images and the trajectories of the ankle joints. The trajectory in the sagittal (y-axis and movement of rostral/caudal directions) was calculated using the center of the hip as the zero (0) point. C: In the trajectories of the ankle joints in the frontal view (z-axis and movement of dorsal/ventral directions), the zero (0) point was the running belt. Right and left panels illustrate the right and left ankle, respectively. The stance (gray) and swing (white) phases in both limbs are indicated. Red, blue, and black lines indicate BD with Ex, BD w/o Ex, and sham w/o Ex, respectively. These trajectories are shown as the mean ± SEM. Bonferroni’s correction was used for post hoc comparisons when the ANOVA revealed significant differences. The comparison of the sham w/o Ex group and BD w/o Ex group; the significant difference is shown as p < 0.05 (*) and p < 0.01 (**). The comparison of the sham w/o Ex group and BD with Ex group; the significant difference is shown as p < 0.05 (¢) and p < 0.01 (¢¢). The comparison of the BD w/o Ex group and BD with Ex group; the significant difference is shown as p < 0.05 (†) and p < 0.01 (††).

Fig. 5. The cyclogram area analysis on the horizontal, sagittal, and frontal planes in the one-step cycle. The areas of the cyclogram were figured by the ankle during the one-step cycle, using the initial 5th finger contact as the zero (0) point. A: The area of the cyclogram and the illustrating view of the stick figure of mice on the horizontal (x-y) plane. B: The area of the cyclogram and the illustrating view of the stick figure of mice on the sagittal (y-z) plane. C: The area of the cyclogram and the illustrating view of the stick figure of mice on the frontal (z-x) plane. Arrow indicated the movement of the ankle bead marker. Red, blue, and black line/bar indicated BD with Ex, BD w/o Ex, and sham w/o Ex, respectively. The results of the cyclogram area are shown as a bar graph. These cyclograms and bar graph are shown as the mean ± SEM. Bonferroni’s correction was used for post hoc comparisons when the ANOVA revealed significant differences. In the comparison of the sham w/o Ex group and BD w/o Ex group, the significant difference is shown as p < 0.05 (*) and p < 0.01 (**). In the comparison of the sham w/o Ex group and BD with Ex group, the significant difference is shown as p < 0.05 (¢) and p < 0.01 (¢¢). In the comparison of the BD w/o Ex group and BD with Ex group, the significant difference is shown as p < 0.05 (†) and p < 0.01 (††).
significantly different compared with that in both the sham w/o Ex and BD with Ex mice.

On the frontal plane (z-axis and movement of dorsal/ventral directions; Fig. 4C), the trajectories of the right ankles in the sham w/o Ex and BD with Ex mice were similar, going up during the stance phase and down in the swing phase. However, the BD w/o Ex mice was significantly different from the trajectories in both the sham w/o Ex and BD with Ex mice, showing a higher position and less up-and-down movement during the one-step cycle. This indicated that BD w/o Ex mice were standing on tiptoes and could not raise their foot high during the swing.

**Fig. 6.** Kinematic analysis of the dorsal/plantar flexions in ankle joints during one-step cycle. A: The criteria of dorsiflexion (D/F) and plantar flexion (P/F) of the ankle joints. D/F and P/F were defined as the movement of the fifth toe in the direction of the knee (180°) or the direction of the treadmill belt (0°), respectively. B: Illustration of the D/F and P/F movement during the one-step cycle with the stick mouse figure. The stance (gray) and swing (white) phases in both limbs are indicated below. C: The trajectory of the angle of the ankle joint. D: The trajectory of angular velocity at the ankle joint. E: The trajectory of angular accelerations at the ankle joint. Right and left figures depict the right and left ankle, respectively. Red, blue, and black line indicated BD with Ex, BD w/o Ex, and sham w/o Ex, respectively. These trajectories are shown as the mean ± SEM. Bonferroni’s correction was used for post hoc comparisons when the ANOVA revealed significant differences. In the comparison of the sham w/o Ex group and BD w/o Ex group, the significant difference is shown p < 0.05 (*) and p < 0.01 (**). In the comparison of the sham w/o Ex group and BD with Ex group, the significant difference is shown as p < 0.05 (¢) and p < 0.01 (¢¢). In the comparison of the BD w/o Ex group and BD with Ex group, the significant difference is shown as p < 0.05 (†) and p < 0.01 (††).
phase. In the left ankles, the trajectories of all groups did not differ significantly.

These results revealed that the BD impaired the gait of the right limbs and influenced the left limb movements, probably due to compensation.

2.5. Cyclogram area analysis of the ankle

Next, cyclogram areas were compared in the ankle trajectories of the three groups to examine the ankle coordination. On the horizontal (x-y) plane, the cyclogram areas in the ankles of both limbs decreased in BD w/o Ex mice, but the difference was not significant among the three groups (Fig. 5A). On the sagittal plane (y-z), the cyclogram areas in the ankle of the right limbs of both BD groups were significantly smaller than those of sham w/o Ex mice, although the area in the left ankle was not significantly different among the three groups (Fig. 5B). On the frontal (z-x) plane, the cyclogram areas in both ankles of the sham w/o Ex mice were similar and almost round. The area in the ankle of BD w/o Ex mice was smaller and that of the right ankle was irregular than that in the sham w/o Ex group. The direction to the dorsal length in BD with Ex mice was short, same as in BD w/o Ex mice; the direction to the lateral length was longer than that in sham w/o Ex mice, and the shape was a horizontally long oval, although the area was similar to that of sham w/o Ex mice. The cyclogram areas in both limbs decreased in BD w/o Ex mice, and it was significantly smaller in the right limbs comparing to that of the sham w/o Ex and BD with Ex groups (Fig. 5C).

2.6. Kinematic analysis of the ankle joint dorsal/plantar flexion

The gait and trajectory analysis of ankles suggested that the animals subjected to BD had distorted posture and impaired ankle coordination. We then analyzed the movement of the dorsal/plantar flexions of ankle joints. Dorsiflexion (D/F) and plantar flexion (P/F) of the ankle joints were defined as the movement of the fifth toe in the direction of the knee (180°) or the direction of the treadmill belt (0°), respectively (Fig. 6A). The angles of the ankle joints observed on sagittal plane showed a bimodal pattern, which had a smaller peak during the stance phase and a larger peak during the swing phase (Fig. 6B). The angles of the ankle joint in the right limbs showed a significant difference in P/F in both stance (17.5%–46.5%) and swing (82.5%–92.5%) phases between the BD with Ex and BD w/o Ex mice. However, there were no significant differences between these two groups compared with the sham w/o Ex mice (Fig. 6C, right ankle). This and the result of Fig. 4C suggest that the BD w/o Ex mice showed a tendency to be in a planter flexion position during the stance phase, i.e., they were on their toes. While the angles of the ankle joints in the left limbs were insignificantly different between the mice in sham w/o Ex mice and in BD with Ex mice in the one-step cycle, they were significantly different with those of the BD w/o Ex mice. The angles of the swing phase, which consisted of the stance phase of the right limb, in the BD w/o Ex mice were significantly smaller than those of the sham w/o Ex mice (17.0%–47.5%) and the BD with Ex mice (35.5%–48.0%) (Fig. 6C, left ankle), suggesting that the mice in BD w/o Ex might be compensating for the impairment of the right side.

The angular velocity in right and left limbs also exhibited significant differences among the groups. The velocities of the right ankle joints demonstrated significant differences between the sham w/o Ex and BD with Ex mice (62.5%–66.0%), sham w/o Ex and BD w/o Ex mice (66.5%), and BD with Ex and BD w/o Ex mice (57.0%–62.0%, and 83.0%–87.0%). Interestingly, the differences in velocity were concentrated in the timing of the transition from stance to swing phase (Fig. 6D, right ankle). These are similar to the velocities of left ankle joints. The velocities of the left ankle were significantly different between the sham w/o Ex and BD w/o Ex (47.0%–49.5%), and BD with Ex and BD w/o Ex (36.5%–39.0%, and 47.5%–50.0%). These were consistent with the timing of the transition from the stance to swing phase of the left limbs (Fig. 6D, left ankle).

The angular accelerations of the right ankle joint indicated that mice in the BD w/o Ex group began acceleration earlier than did the other groups, exhibiting a significant difference to BD with Ex (45.5%–53.0%, and 63.5%–67.0%) (Fig. 6E, right ankle). The angular velocity and acceleration of the left ankle joint were not significantly different among the groups because of a larger deviation. However, the trajectory of acceleration in the BD w/o Ex mice was quite different from that in the sham w/o Ex and BD with Ex mice (Fig. 6E, left ankle).

2.7. Left hemidecortication and the damage of the right corticospinal tract

Finally, we confirmed the incidence of BD among the groups. The brain of sham w/o Ex mice did not demonstrate obvious cortical damages (Fig. 7A, D). However, the BD w/o Ex (Fig. 7B, E) and BD with Ex (Fig. 7C, F) mice exhibited similar extensive lesions in the left cerebral cortex. Then, immunostaining of PKCγ in the upper cervical spinal in the sham w/o Ex were clearly recognized in both hemispheres of the corticospinal tract (cst) (Fig. 7G). The immunopositive reactions in both the BD w/o Ex and BD with Ex groups were recognized only in the left hemisphere of the cst and were not observed in the right hemisphere (Fig. 7H), indicating a lack of neural connection in the right cst by left hemidecortication.

3. Discussion

In the present study, we examined the effect of mild-intensity running exercise on the gait and movement of ankle joints in mice after BD using 3D-based motion capture techniques. We determined that the mice subjected to mild-intensity running exercise improved their gait compared with mice w/o exercise. We also determined that BD w/o Ex mice walked with compensation of nonaffected limbs.

3.1. Mild-intensity running exercise after brain injury

Various running exercise intensities have been shown to have different influences on motor functional recovery and neuroprotection after BD (Morishita et al., 2020; Pin-Barre et al., 2017). To determine mild running exercise intensities as rehabilitation stress in the present study, we evaluated the exercise tolerance and the inflection point by measuring VO2 and VCO2. The evaluation of the VO2 and VCO2 during exercise is widely used both rodents and human studies (Balady et al., 2010; Picoli et al., 2018) and is a relatively simple approach (Voss et al., 2013). Herein, we subjected mice to running adaptation for a few days before the incremental running test. A previous report concluded that pre-training is required for at least 2–3 weeks to exert neuroprotection after ischemic stroke (Zhang et al., 2011). Therefore, we considered that the effect of recovery on brain damage by the primary physical fitness assessment had little influence on brain protection during surgery and motor functional recovery.

3.2. Improvement of mild-intensity running exercise on the gait after brain injury

Four weeks after BD, we compared gait parameters and determined that the BD w/o Ex mice showed a significant impairment of parameters for steps and were suggested to have a distorted posture. These heterogeneous gait, such as a reduction in step length and extension in stride width, were supported by findings in stroke patients (Chen et al., 2005; Hak et al., 2013). We also observed an obvious compensation of the unaffected limb to maintain the posture of the mice.

To explain the heterogeneous gaits in detail, we determined the trajectory and the cyclogram of the ankle. The trajectory analysis traces a marker and determines the disturbance in the gait. The cyclogram is able to determine dynamic or arrhythmic movements of the step (Awai and Curt, 2014; Goswami, 1998; Park et al., 2021). The trajectory analysis determined that BD mice positioned their ankles on the inside as compared with sham w/o Ex mice. Indeed, the ankle in BD w/o Ex mice
was always positioned higher and with less up-and-down movement during the gait. This suggested walking on an equinus foot in the BD w/o Ex mice. These could represent impaired ankle movements similar to those of stroke patients (Kinsella and Moran, 2008; Li, 2020). These are improved by mild-intensity running exercise.

By the cyclogram analysis, less dynamic and flexible movements in BD w/o Ex mice were suggested by the smaller cyclogram area. However, these also recovered after mild-intensity running exercise for 4 weeks. The less dynamic movements and the recovery were confirmed by the angle, velocity, and acceleration of the ankle joints. These analyses indicate the mobility, flexibility, and dynamism of joints. In the present analyses, the differences were concentrated on the right side, the motion was fixed at P/F in the stance phase, and the shift was restricted to D/F in the swing phase. These results suggest that the ankle joint in the BD w/o Ex mice lacked mobility due to the damage and could not respond flexibly to the gait rhythm. In addition, velocity and acceleration in the BD w/o Ex mice also exhibited significant differences from those of the other groups. These differences in the velocities of both limbs were concentrated on the timing of the transition from stance to swing phases, suggesting the BD w/o Ex mice had difficulty switching from bucking on the floor (required for the motion of P/F) to swinging the leg forward (accompanying D/F). This timing is consistent with the loading response to pre-swing in the human gait (Stoeckl et al., 2015). It has been reported that stroke patients with gait disturbances have impairment in D/F and P/F (Lin et al., 2006). From these findings, an equinus walk was observed in the BD w/o Ex mice, and this walk recovered in the BD with Ex mice. More interestingly, in the unaffected limbs, BD w/o Ex mice showed significant impairment in D/F in the swing phase, consistent with the timing of P/F in the stance phase in the affected limbs of both the sham w/o Ex and BD with Ex groups. This again suggests that the unaffected limbs in the BD w/o Ex mice maintained the balance and coordinated the motion of running. Our findings also indicate the need to analyze the motion of the unaffected limbs as well as the affected limbs.

3.3. Importance of kinematic analyses and further research

Rodents with quadruped walking are considered difficult to evaluate using locomotor analyses, and thus, it has not been possible to evaluate these animals in the clinical setting of rehabilitative therapy because local or small behavioral impairments are hard to assess, and they also have different gaits. Using a 3D-motion capture technique combined with kinematic analysis, we quantitatively demonstrated the impairment of the joint mobility and flexibility and the partial recovery after BD with and without mild-intensity running exercise as a rehabilitative therapy. Our results also suggest the importance of evaluating the compensation from the nonaffected contralateral limbs. The results including former publications using kinematic analyses (DiGiovanna et al., 2016; Kawai et al., 2015; Miri et al., 2017; Ueno and Yamashita, 2011; Ueno et al., 2018) strongly suggest the application of kinematic analyses in rodent experiments as well as in clinical studies.

Conversely, we did not examine whether or how gait movements of the hindlimbs recovered after mild-intensity running exercise. Several studies have demonstrated that exercise protects against brain damage through the promotion of angiogenesis, mediation of the inflammatory response, inhibition of glutamate over-activation, protection of the
blood-brain barrier, and inhibition of apoptosis (Liu et al., 2022; Zhang et al., 2011). In addition to these protection phenomena, there are putative mechanisms such as neuronal or axonal regeneration from residual and/or contralateral neurons (El-Sayes et al., 2019; Ishida et al., 2019; Maier et al., 2008; Morishita et al., 2020; Wang et al., 2020) that explain the recovery of neuronal function after a rehabilitative therapy. Loss of muscular power and contracture also might contribute to the impairment in the BD w/o Ex mice (Hesse et al., 1994, 1996).

Moreover, we could not determine how much intensity was “appropriate” as rehabilitative therapy. Further studies need to evaluate physical and molecular/cellular recoveries after rehabilitative therapy in basic experiments including animal studies, which will bring hope to suffering patients.

3.4. Conclusion

In this study, we evaluated the motor dysfunction and recovery of mice after BD with/without aerobic running exercise using 3D kinematic analysis. Our results suggest that the 3D-kinematic analysis will become a powerful tool for detecting minor behavioral alterations in the impairment of the affected side as well as the compensation from the unaffected side.

4. Methods

4.1. Animals and husbandry

Male C57BL/6J mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). Mice were subsequently bred in our animal care facility under conventional holding conditions, housed in cages with 12-h light/dark cycles (lights were turned on at 8:00 AM), and received water and food (LabDiet 5058) ad libitum. All animal care and experimental procedures were approved by and followed the Institutional Animal Care and Use Committee of Showa University (approval number, 08029, 09013, and 02055).

4.2. Protocol overview

Mice (8 weeks old, weighing 22.9 ± 0.5 g) were randomly assigned to one of the three experimental groups as follows: brain damage (BD) with treadmill running exercise (Ex) (BD with Ex, n = 10), BD without Ex (BD w/o Ex, n = 8), sham-operation without Ex (sham w/o Ex, n = 8). A schematic illustration of the experimental design is shown in Fig. 1A. Briefly, we assessed the physical fitness of mice before subjecting them to either left hemidecortication as BD or the sham operation. Beginning one day after the surgery and continuing for 4 weeks, BD with Ex mice performed 30 min of treadmill running five times/week. Mice in the BD w/o Ex and sham w/o Ex groups were maintained in normal animal cages for 4 weeks.

4.3. Treadmill chamber equipment: the measurement of VO2 and VCO2 during treadmill running exercise

The mice (MK-680AT/02M, Muromachi Kikai Co., Ltd., Tokyo, Japan) were compulsively exercised on an airtight treadmill (width × depth × height of 70 × 440 × 75 mm; 5° upslope) while receiving air continuously at 1.0 L/min by ventilation to obtain oxygen/carbon dioxide (O2/CO2) pulmonary gas metabolism measurement (Oxymax ver 4.7x, Columbus Instruments, Columbus, OH) as previously described (Izumizaki et al., 2013) (Fig. 1B).

4.4. Primary physical fitness assessment

Previous studies have shown that different intensities of running exercise resulted in different recovery of motor functions after brain injury (Morishita et al., 2020; Pin-Barre et al., 2017). Therefore, to evaluate the physical fitness of the mice, we first examined the running exercise test, which is popularly used in animal studies (Ayachi et al., 2016; Hoya et al., 2007; Picoli et al., 2018; Pin-Barre et al., 2017), and determined the ability and capacity of exercise tolerance in mice. Simultaneously, the pulmonary gas exchange of O2 and CO2 were measured to estimate the exercise intensity level. They were expressed as O2 consumption (VO2 ml/min/kg), and CO2 production (VCO2 ml/min/kg). The respiratory exchange ratio (RER) was calculated by dividing VCO2 by VO2 (Fig. 1B).

By the day before the incremental running test, BD with Ex mice were acclimatized to treadmill running a few times for 30 min at a speed of 10 m/minute due to pre-training. On the day of the test, the mice were placed in a treadmill chamber for 30 min for normalization of the respiratory environment followed by measurement of VO2 and VCO2 for 5 min in the resting condition.

Mice were next forced to perform the running exercise. The increasing speed of treadmill rotation was 1, 3, 5, 10, 15, and 20 m per minute for first 6 min. After reaching speed at 20 m/minute, the treadmill rotation further increased the speed at 2 m/minute by the minute (Fig. 2A) and continued to increase until the mice dropped out (Fig. 2B). The exercise tolerance in each mouse was determined by the point that the mice could not follow the treadmill rotation. The VO2 and VCO2 were collected every 10 s and expressed as the average for every 1 min.

To determine if the rate of increase in VCO2 exceeds the rate of increase in VO2 (Beaver et al., 1986), the inflection point was examined by based on the correlation between VO2 and VCO2. The inflection point was analyzed using piecewise regression and two-segmented linear models with the regression library of SigmaPlot (SigmaPlot 13; Systat Software Inc, San Jose, CA) (Izumizaki et al., 2011; Tsukada et al., 2017).

4.5. Left hemidecortication BD model

Mice in the BD with Ex (n = 10) and BD w/o Ex (n = 8) groups were subjected to left hemidecortication according to our previous report (Yoshikawa et al., 2014). Briefly, the mice were anesthetized with 2.5 %-3.5 % sevoflurane inhalation via a face mask and fixed in a stereotaxic frame in the prone position. Under aseptic conditions, an incision was made along the midline of the head, and the cranial bone was exposed. The cranial bone on the sensorimotor area (caudal 3 mm and lateral 3 mm from the bregma level toward the frontal pole) was then carefully drilled to expose the left cortical surface (Pronichev and Lenkov, 1998; Tennant et al., 2011). The left neocortical parenchyma was aspirated carefully using a microopette tip connected with an aspirator. After left cortical aspiration, the cranial bone was returned to the original position, and the skin was closed with 4-0 silk suture. The mice were returned to their home cages. Sham w/o Ex mice (n = 8), as a control for all aspects of the experimental surgery, underwent the same surgical procedure except for the aspiration of neocortex.

4.6. Running exercise

One day after BD surgery, mice in the BD with Ex group (n = 10) were made to perform the running exercise for rehabilitation on the treadmill (10 m/minute for 30 min, once a day, 5 times/week) for a total of 4 weeks using the treadmill equipment as described (Fig. 1B). The monitoring of VO2 and VCO2 in BD with Ex animals was conducted for times at 1 (day 8), 2 (day 15), 3 (day 22), and 4 (day 29) weeks (at 7-day intervals) after the BD. No mice dropped from the experiments.

4.7. The 3D-motion capture analysis

4.7.1. Stick color marker beads on mice joints and recording the gait

One month after the BD or sham operation, the gait and movement of the mice were evaluated by 3D-based motion capture technology. Following inhalation 3 % sevoflurane in air as anesthesia, a total of
14 color beads were bound to the shaved shoulders, elbows, wrists, hips, knees, ankles, and base of little fingers in the four limbs of the mice (Fig. 1C). After an approximate 30-min recovery period from anesthesia, the mice were made to run on the treadmill at 10 m/min, and their movement was recorded for at least ten continuous steps with four synchronized digital cameras with a resolution of 1904 × 822 at 120 Hz (EX-100PRO, Casio Computer Co., ltd, Tokyo, Japan). The cameras were positioned in different places (right-anterior, right-posterior, left-anterior, left-posterior) around the treadmill apparatus so that a color bead was captured by at least two cameras (Fig. 1D).

4.7.2. Gait analysis with the KinemaTracer system®

The KinemaTracer system® (KISSEI COMTEC, ltd. Nagano, Japan) (Tajino et al., 2018; Ueno and Yamashita, 2011) was used to analyze the 3D gait movement via the tags on the color bead markers. In brief, every marker bead was traced automatically by color, and a stick figure was conceptualized by connecting the beads. Moreover, the center of the hip was defined between two hip marker beads of both hindlimbs (Fig. 1E). The quantifications of 3D-position of the marker beads were computed with a pre-captured cuboidal calibration box (5 × 20 × 20 cm).

In the general gait evaluation, one-step cycle (s), stance phase (s), swing phase (s), and double-stance phase (s) were analyzed by calculating the 5th finger markers.Stride length (cm), step length (cm), and stride width were analyzed by calculated ankle markers. The stride width was analyzed in detail from the center of the hip to both sides of the ankle marker and 5th finger markers.

In the trajectory analysis of the ankle marker, the center of hip was standardized as the zero (0) point in the horizontal and sagittal plane, and the running belt was standardized as the zero (0) point in the frontal plane.

The cyclogram areas were calculated from the trajectories of each step with MATLAB (R2016B; MathWorks Inc., Natick, MA) on a computer running Windows 10 with standardized data (Awas and Curt, 2014; Goswami, 1998). In this evaluation, the cyclogram areas were drawn from the first to the second touch of the 5th finger on the running belt and analyzed.

The angles, angle velocities, and angle accelerations of the ankle joints were measured automatically from the trajectory of the knee, ankle, and 5th finger with the KinemaTracer system® on the sagittal plane.

4.8. Confirmation of BD and immunostaining

To validate the left hemidecortication, the brain injury was macroscopically observed with coronal sections, and the upper cervical spinal cord was immunostained with an antibody against PKCγ. We previously reported that PKCγ immunoreactions, which can usually be recognized in the corticospinal tract (Malmberg et al., 1997; Mori et al., 1990), are decreased after neocortical injury in neonatal and adult rodents (Yoshikawa et al., 2011, 2014). After 3D kinematic evaluation, the mice were deeply anesthetized with sodium-pentobarbital (100 mg/kg, i.p.) and transcardially perfused with 4 % paraformaldehyde (PFA) in a 0.1 M phosphate buffer (PB, pH 7.2) following saline. The brain and cervical spinal cord were carefully removed, and were post-fixed in 4 % PFA overnight at 4 °C. The brains were then cut into approximately 2-mm thick coronal slabs from 2 mm to −2 mm of bregma, and the cortical injury was macroscopically observed in the anterior surface.

The cervical spinal cords were immersed in 20 % sucrose in 0.1 M PB for cryoprotection and embedded using an embedding solution (20 % sucrose in PB: O.C.T. compound (Sakura Finetech, Tokyo, Japan): 2:1) with liquid nitrogen-cooled isopentane. Ten micrometer thickness coronal sections were immunostained for PKCγ. After washing with PBS, the sections were immersed in 0.3 % H2O2 for 30 min to quench internal peroxidase reactions and were incubated with 2 % normal horse serum (NHS; Vector Laboratories, Burlingame, CA) to block nonspecific antibody reactions. The specimens were then incubated with polyclonal rabbit antibody against PKCγ (1:1000; Santa Cruz biotechnology, Santa Cruz, CA) overnight at 4 °C. Subsequently, after washing with PBS, the sections were incubated with biotinylated goat anti-rabbit IgG secondary antibody (1:400; Thermo Fisher Scientific, Waltham, MA) and visualized in an avidin-biotin complex solution (Vector Laboratories, Inc., Burlingame, CA) followed by diaminobenzidine (DAB; Sigma, St. Louis, MO) as a chromogen. The sections were observed with the aid of light microscopies, such as AX70 (Olympus, Tokyo, Japan) and BZ-700 (Keyence Co ltd., Tokyo Japan).

4.9. Statistical analysis

All the values are reported as mean ± SEM values. VO2, VCO2, and RER at each week, general gait parameters, the trajectory of ankle, the cyclogram area, and the movement of the ankle joint were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni’s correction between the BD with Ex, BD without Ex, and sham without Ex groups. Bonferroni’s correction was used for post hoc comparisons when the ANOVA revealed significant differences. This statistical analysis of VO2, VCO2, and RER at each week, general gait parameters, and the cyclogram area performed using SPSS version 25 (IBM Corp., Armonk, NY, United States). This statistical analysis in the trajectory of ankle, and the movement of the ankle joint was calculated on MATLAB (R2016B; MathWorks Inc., Natick, MA, United States) on a computer running OS X El Capitan. All p values<0.05 were considered statistically different.

Author contributions

AY and HO proposed this study design. AY did all the experiments and wrote this article. ShK measured and analyzed the metabolism in mice running on the treadmill data. KM, KH, and MY analyzed the mouse’s ankle movement using 3D kinematical method during running on treadmill. KM and SaK performed the immunohistochemistry. NK made the script for Matlab and did the statistical analysis. AY, HO, and MI were responsible for drafting the article. All authors revised the article and provided approval for publication of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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CRediT authorship contribution statement

Akira Yoshikawa: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Validation, Visualization, Writing - original draft, Writing - review & editing. Hirokazu Ohtaki: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Software, Validation, Visualization, Writing - review & editing. Kazuyuki Miyamoto: Formal analysis, Investigation, SungHyek Kim: Formal analysis, Investigation, Methodology. Kazunori Hase: Formal analysis, Investigation, Methodology. Makoto Yoshida: Formal analysis, Investigation, Methodology. Shotaro Kamijo: Investigation, Methodology. Sawa Kamimura: Investigation, Methodology. Nobuyoshi Koiva: Formal analysis, Software. Masahiko Izumizaki: Resources, Supervision, Writing – review & editing.
Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability
No data was used for the research described in the article.

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Appendix A. Supplementary data

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