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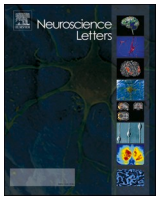

In vivo bioluminescence imaging revealed the change of the time window of BDNF expression in the brain elicited by a single bout of exercise following repeated exercise

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ABSTRACT

Exercise increases the expression of brain-derived neurotrophic factor (BDNF) in the brain and contributes to cognitive and sensorimotor functions. This study aimed to elucidate how repeated exercise modifies BDNF expression elicited by a single bout of exercise in the brain using in vivo bioluminescence imaging (BLI). Bdnf-luciferase (Luc) mice with the firefly *luciferase* gene inserted at the translation start point of the *Bdnf* gene were used for BLI to monitor changes in BDNF expression in the brain. The treadmill exercise at a speed of 10 m/s for 60 min was repeated 5 days a week for 4 weeks. BLI in individual subjects was repeated four times: before the exercise intervention, on the first exercise day, and 14 and 28 days after the start of the intervention. Each BLI was performed after a single bout of exercise and monitored for 8 h after exercise. Repetitive BLI showed that the exercise regimen enhanced BDNF expression in the brain, specifically at 4–8 h after a single bout of exercise. Repeated exercise for 2 weeks accelerated the start of enhancement after a single bout of exercise, but not after 4 weeks of repeated exercise. This study showed that repeated exercise modulated the time window of exercise-enhanced BDNF expression, suggesting that repeated exercise could change the sensitivity of gene expression to a single bout of exercise. These findings can be attributed to the advantages of in vivo BLI, which allowed us to precisely measure the time course of BDNF expression after repeated exercise in individual subjects.

1. Introduction

Brain-derived neurotrophic factor (BDNF) is a neurotrophin that induces various neural functions, including neuronal survival, differentiation, and plasticity in the central nervous system (CNS) [1]. BDNF expression in the brain contributes beneficially to the cognitive, psychiatric, and sensorimotor functions that the CNS controls [2]. In particular, BDNF expression in the brain improves learning and memory [3,4] and prevents cognitive deficits caused by aging and degenerative diseases, such as Alzheimer's disease [5].

It has been well recognized that aerobic exercise enhances BDNF expression in the brain [6]. Therefore, researchers have focused on the therapeutic effects of exercise-enhanced BDNF expression on CNS disorders associated with aging and degenerative diseases. To date, several interventions that involve aerobic exercise have been encouraged in older adults and have shown beneficial effects on cognitive function [7]. Previous studies based on mRNA expression analyses or protein assays using rodent brain samples have shown that a single bout of exercise induces temporal BDNF upregulation [8,9]. Focusing on temporal exercise-enhanced BDNF expression, Rasmussen et al. showed that BDNF expression was induced 2–6 h after a single bout of exercise in both the hippocampus and cortical motor cortex [10], while Huang et al. showed enhanced expression in the hippocampus 2 h after exercise [11], suggesting that there is a time window for exercise-enhanced BDNF expression.

Recently, with the development of genetic engineering, it has become possible to visualize gene expression in vivo using bioluminescence imaging (BLI) systems in gene-inserted mice. Fukuchi et al. (2015)

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developed Bdnf-Luc mice to visualize BDNF expression in live mice [12]. Bdnf-Luc mice contain the *Bdnf-Luc* gene, in which a *luciferase* gene is inserted at the start of translation of the *Bdnf* gene [13,14]. Because luciferase is expressed under the control of the *Bdnf* gene promoter, the luminescence associated with *Bdnf* gene expression can be detected by administration of a luciferase substrate, Akalumine-HCl (TokeOni) [14]. In vivo BLI using Bdnf-Luc allows us to track the dynamics of BDNF expression repeatedly in the same individual [13]. Our previous study using BLI showed that BDNF expression was significantly enhanced during 1–3 h after a single bout of exercise in the same individual [15], and quantitative PCR and protein assays for BDNF showed temporal enhancement [8–11,16].

Specifically, daily exercise or exercise habits have been widely recommended to prevent neurological disorders, maintain neurological systems, and enhance neuroplasticity [6,7]. Limited studies using rodent brains have shown that repeated exercise upregulates BDNF expression in the hippocampus more abundantly [17] and extends the time window of BDNF expression in the hippocampus and cerebellar cortex after a single bout of exercise [18,19]. Although repeated exercise could be expected to modulate temporal exercise-enhanced BDNF expression in the brain, modulation of the precise time course, including the start time and duration of BDNF expression, after a single bout of exercise over repeated exercise is not well understood. When examining the time course of BDNF expression after exercise, it is ideal to track its

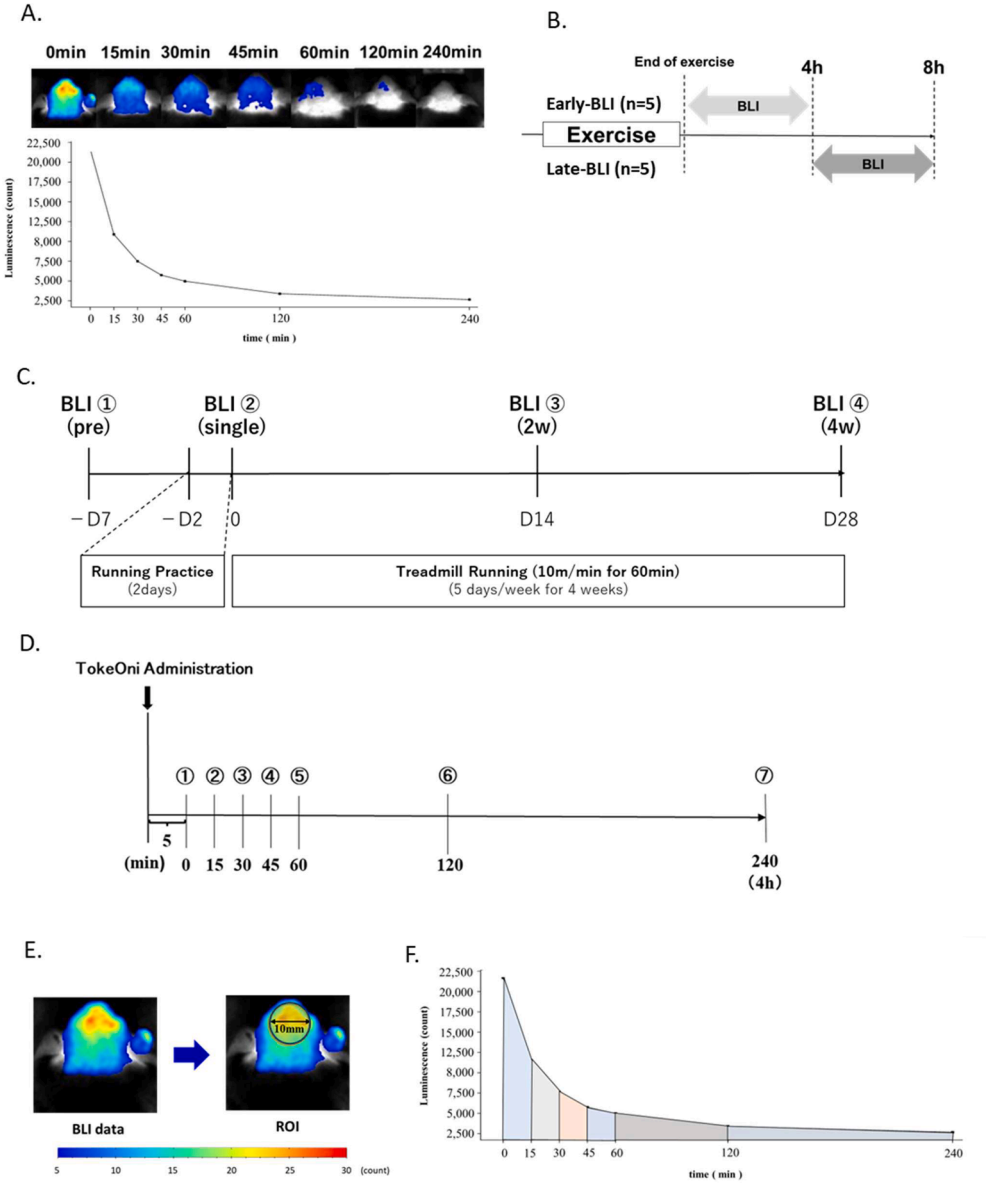

Fig. 1. Experimental procedure. (A) Representative time course of the luminescence for 240 min after the administration of TokiOni. (B) Assignment of mice to two groups based on the timing of BLI after exercise. Mice are divided into two groups: an early-BLI group, in which BLI is performed between 0 and 4 h after the end of exercise, and a late-BLI group, in which BLI is performed between 4 and 8 h after exercise. (C) The days of experiment for BLI during the exercise intervention for 4 weeks. BLI is performed on a total of four measurement days: 1 week before the start of exercise (pre), after a single bout of exercise session (single), 2 weeks after the start of repeated exercise (2w), and after the end of all exercise interventions (4w). The exercise intervention is conducted at a frequency of 5 days a week for 4 weeks, with practice on the previous 2 days. (D) BLI time points on an experiment day on each measurement day. A total of seven BLIs are taken over a total of 240 min (4 h), starting 5 min after TokeOni administration. (E) ROI setting. A circular ROI with a diameter of 10 mm is set up based on the brain atlas of C57BL/6 strain mice to specify and detect luminescence from the brain region. (E) Calculation of the cumulative BLI for 4 h. (F) Calculation of cumulative BLI for 240 min (4 h) based on numerical integration using the trapezoidal approximation from a time-point plot at 15, 30, 45, 60, 120, and 240 min after a single administration of TokeOni, showing the luminescence intensity over 240 min.

expression in the same individual subject at multiple measurement points throughout the exercise intervention. However, in conventional quantification of BDNF expression in laboratory animals, it is necessary to use postmortem brain samples for biochemical analyses, making it difficult to track the time course of exercise-dependent BDNF expression in the same individual repeatedly or to measure BDNF expression in the living brain in vivo. In vivo BLI is expected to overcome these limitations regarding repeated analyses of an individual and provide a precise understanding of the time course of BDNF expression over repeated exercise.

The objective of this study was to precisely visualize the effect of repeated exercise on modulation of BDNF expression induced by a single bout of exercise in the brain using in vivo BLI. Therefore, this study aimed to clarify the effects of repeated exercise interventions on BDNF expression over time by tracking the same individuals during the intervention.

2. Methods

2.1. Animal and experimental procedures

Ten adult male Bdnf-Luc mice derived from C57BL/6 (8–9 weeks old, 24.2 ± 1.2 g, mean ± S.D) were used in this study [12–14]. A previous study showed that BDNF expression in the brain, detected by BLI using TokeOni, was enhanced significantly within 1–3 h after vigorous exercise and tended to increase thereafter [15]. The luminescence gradually decayed within 4 h after substrate administration (Fig. 1A), consistent with a previous study [15]. Furthermore, the luminescence level immediately after TokeOni administration was inhibited, indicating that sufficient intermission for more than 24 h is required for subsequent BLI using TokeOni [14]. Considering these limitations and characteristics of BLI using TokeOni, the mice were assigned to two groups to monitor BLI for a total of 8 h after exercise (early-BLI group (n = 5), in which BLI was performed between 0 and 4 h after the end of exercise and late-BLI group (n = 5), in which BLI was performed between 4 and 8 h after exercise; Fig. 1B). All mice were housed in a temperature- and humidity-controlled room on a 12-h light/dark cycle with food and water available ad libitum. All study procedures were approved by the Ethics Committee for Animal Research of Hokkaido University, Japan, and were carried out according to the guidelines of the committee. This study was conducted with the approval of proliferation prevention measures for type 2 use based on the application of genetic recombination experiments at Hokkaido University.

2.2. Aerobic exercise

A 4-week exercise intervention was performed in all mice 5 days a week (Fig. 1C). Based on a previous exercise intervention protocol that enhanced the expression of BDNF and neural activity markers in the brains of healthy mice, mice exercised on a treadmill (MK-680, Muromachi Kikai, Japan) at a speed of 10 m/min for 60 min [20,21]. The intensity used here (10 m/min), approximately 60 % V_{O2max} [20], is recognized as appropriate for exercise-enhanced BDNF expression [6]. Before starting the exercise intervention, mice ran on a treadmill at a speed of 10 m/min for 20 min for 2 days to adapt to the exercise environment [22].

2.3. In vivo BLI

All mice in the early and late-BLI groups were subjected to a total of four BLI sessions: 1 week before the start of exercise (pre), after a single bout of exercise intervention (single), 2 weeks after the start of exercise intervention (2 weeks, 2w), and after the completion of all exercise interventions (4 weeks, 4w) (Fig. 1C).

BLI was performed approximately 240 min (4 h) after TokeOni administration. BLI was performed in approximately 0 min to 4 h after exercise in the early-BLI group and within approximately 4–8 h after exercise in the late-BLI group. One day before BLI, the black hair on the top of the head of the mice was shaved under inhalation anesthesia with 2.0 % isoflurane. TokeOni, the luciferase substrate, was dissolved in saline at a concentration of 10 mg/ml. Mice were anesthetized by inhalation of 2.0 % isoflurane and then TokeOni was administered intraperitoneally (0.1 ml TokeOni/10 g body weight [substrate dose: 100 mg/kg]), according to a previous study [14]. TokeOni was administered immediately after each bout (Fig. 1D). Five minutes after TokeOni administration, the first BLI was performed using an in vivo imaging system (Photon Imager; Biospacelab, France). Following the first measurement (0 min), we subsequently performed BLIs at 15, 30, 45, 60, 120, and 240 min later (a total of seven measurements after a single administration of TokeOni) as shown in Fig. 1D. Each BLI image was captured for 2 min using a 660 nm optical filter. Pseudocolor luminescence images, representing the spatial distribution of emitted photons, were superimposed on photographs of mice taken in a dark chamber.

2.4. Analyses of BLI

2.4.1. Region of interest (ROI) setting

BLI images were analyzed using M3 vision (Biospacelab, France). An ROI was set up to detect luminescence in the brain region. In this study, a circular ROI with a diameter of 10 mm was set up based on the brain atlas of C57BL/6 mice to specify and detect luminescence from the entire cerebral region according to the mouse brain atlas [23]. The cerebrum was located 3–4 mm anterior and 5–6 mm posterior to the bregma, 10 mm in the longitudinal direction and within 10 mm in the lateral direction. Therefore, an ROI with a 10-mm diameter covers most of the cerebrum. Additionally, BLI using TokeOni has been reported to allow the detection of deep brain regions [14,24]. Therefore, the luminescence detected in this study could be reflected in BDNF expression not only from surface regions, such as the cerebral cortex, but also from deeper regions, such as the hippocampus and basal ganglia. Based on previous studies showing that the luminescence intensity of the brain is particularly strong in BLI using TokeOni as a substrate [14], we determined the position where the total photon count value in the ROI showed the maximum level on the head and recorded this value as the luminescence intensity of the brain for each mouse (Fig. 1E). This analysis was performed by two examiners who were blinded to the assignment of the experimental group and the image acquisition time, and the mean values were used as indicators of BLI.

2.4.2. Analysis 1: Analysis of the cumulative BLI level over 4 h

In analysis 1, to detect total luminescence for 240 min (4 h) after TokeOni administration (Fig. 1D) in four BLI sessions (Fig. 1C), the luminescence level for 4 h after TokeOni administration was calculated for each group based on numerical integration using the trapezoidal approximation from a time-point plot showing the luminescence intensity over 240 min (Fig. 1E). The horizontal axis is the time (0, 15, 30, 45, 60, 120, and 240 min from the first imaging) and the vertical axis is the luminescence level (count), based on the data at seven-time points detected by M3 vision. Numerical calculations were performed using MATLAB R2020b (MathWorks, USA). The data are shown as the luminescence level relative to the mean level of the pre session.

2.4.3. Analysis 2: Analysis of BLI at each measurement time point

In analysis 2, we analyzed the luminescence at each shooting time point to detect the time window of exercise-enhanced BDNF expression. Considering the experimental limitation of the gradual decay of luminescence after substrate administration (Fig. 1A) [14,24], we interpreted the average luminescence level of the pre at each imaging time point as steady-state BDNF expression and analyzed the relative luminescence level over time by setting the average luminescence intensity of the pre at each time point (Fig. 1C). The data are shown as the

luminescence level relative to the mean luminescence at the start of BLI (0 min) of the pre.

2.5. Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics (v26.0, IBM Armonk, NY, USA). Regarding the cumulative bioluminescence level (analysis 1), the luminescence intensity for the cumulative bioluminescence level was processed using a one-way repeated measures analysis of variance (ANOVA). In the case of a significant main effect, post hoc analysis was performed using Dunnett's test with reference to the pretest. Regarding the relative BLI at each measurement time point (analysis 2), the relative luminescence intensity was analyzed using two-way repeated ANOVA with two factors: elapsed time (0, 15, 30, 45, 60, 120, and 240 min) and measurement date (pre, single, 2w, and 4w). When significant main effects or interactions were observed, post hoc analyses were performed using Dunnett's test with multiple comparisons based on 0 min or pre. The significance level was set at 5 %, and data were shown as mean ± S.E.M.

3. Results

3.1. Cumulative BLI level

The procedure for cumulative BLI in each group is shown in Fig. 2A. The cumulative bioluminescence levels are shown in Fig. 2B and 2C. One-way repeated ANOVA did not show a significant exercise-induced modification of the cumulative bioluminescence intensity compared to pre in the early-BLI group ($F_{(3,12)} = 1.836$, $p = 0.194$, $\eta^2 = 0.315$; Fig. 2B). Meanwhile, in the late-BLI group, a significant main effect was observed for measurement day ($F_{(3,12)} = 4.001$, $p = 0.035$, $\eta^2 = 0.500$; Fig. 2B). Dunnett's test showed that luminescence intensity was significantly enhanced after a single bout and 2w exercise compared to pre exercise (pre vs. single: $p = 0.024$, pre vs. 2w: $p = 0.024$, pre vs. 4w: $p = 0.549$; Fig. 2C). Therefore, the analyses of cumulative BLI showed that a single bout of exercise and repetitive exercise for 2 weeks enhanced BDNF expression in the brain, specifically integrated within 4–8 h after exercise.

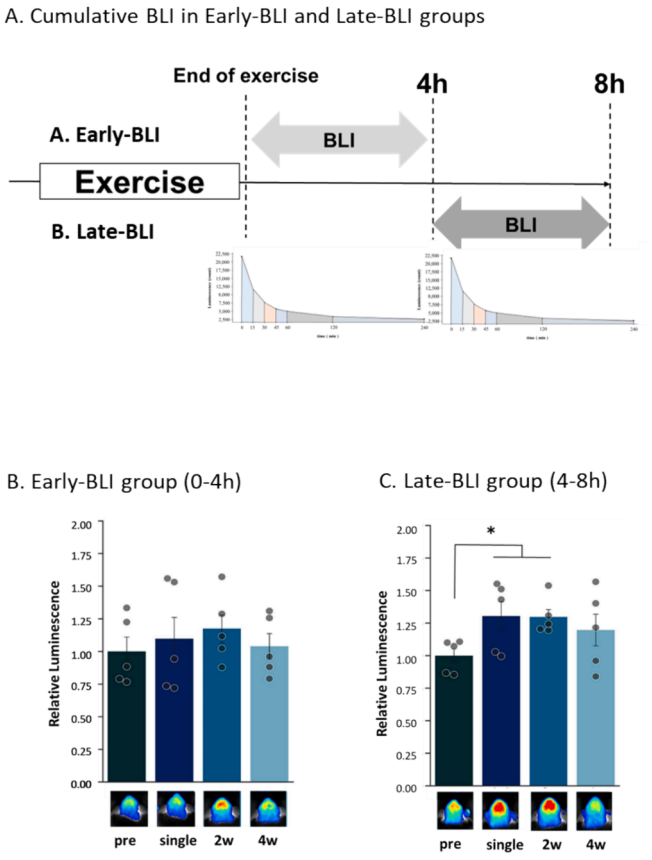

Fig. 2. Effect of exercise on the cumulative BLI level for 4 h. (A) Cumulative BLI for each group. BLI is performed approximately within 0–4 h after exercise in the early-BLI group, while BLI is performed approximately within 4–8 h after exercise in the late-BLI group (B) Cumulative BLI of the early-BLI group. (C) Cumulative BLI in the late-BLI group. Data are shown as mean ± S.E.M. (n = 5 a group). * Indicates a significant difference compared to pre, $p < 0.05$.

3.2. BLI level at each measurement time point

The shooting time points for each group are shown in Fig. 3A. BLI levels at each measurement time point are shown in Fig. 3B and 3C. Two-way repeated measures ANOVA for the early-BLI group (post 0–4 h following exercise) showed a significant main effect of measurement time after the start of BLI ($F_{(6,24)} = 5.828$, $p = 0.001$, $\eta^2 = 0.593$), no significant main effect of measurement day ($F_{(3,12)} = 1.839$, $p = 0.194$, $\eta^2 = 0.315$), and no two-factor interaction ($F_{(18,\ 72)} = 1.224$, $p = 0.264$, $\eta^2 = 0.234$) (Fig. 3B). The post hoc Dunnet's test did not show significant differences; however, the luminescence level tended to be enhanced at 240 min compared to that at the start (0 min) of the imaging ($p = 0.060$).

Two-way repeated measures ANOVA for the late-BLI group (post 4–8 h following exercise) showed a significant two-factor interaction between the measurement day and the measurement time point ($F_{(18,72)} = 1.837$, $p = 0.037$, $\eta^2 = 0.315$; Fig. 3C). In BLI after a single bout of exercise (single), there was a significant enhancement in BLI intensity at 120 and 240 min compared to the intensity at the same time points of pre ($p = 0.027$ and $p = 0.018$). In BLI after 2 weeks of exercise (2w), there was a significant enhancement in BLI intensity at 0, 45, 120, and 240 min compared to the intensity at the same shooting time points in pre ($p = 0.015$, $p = 0.030$, $p = 0.048$, and $p = 0.009$, respectively). In the BLI after 4 weeks of exercise (4w), there was a significant enhancement in the BLI intensity at 240 min compared to the intensity at the same shooting time points in pre ($p = 0.018$). Altogether, a single bout of exercise significantly enhanced BLI 120 min after the start of BLI in the late-BLI group (actually, 6 h after exercise), while 2 weeks of exercise enhanced BLI right after the start of BLI (actually, 4 h after exercise), indicating that repeated exercise accelerated the start time of exercise-enhanced BDNF expression. Meanwhile, acceleration was not detected after 4 weeks of repeated exercise.

4. Discussion

In this study, repetitive BLI analyses within individual subjects showed that the exercise regimen enhanced BDNF expression in the brain, specifically at a later timing, 4–8 h after a bout of exercise, and repeated exercise for 2 weeks accelerated the start of exercise-enhanced BDNF expression in the brain. These findings could be attributed to the advantages of in vivo BLI and the tracking of the time course of exercise-enhanced BDNF expression after repeated exercise in each subject.

To our knowledge, this is the first study to visualize the dynamics of BDNF expression enhanced by repeated exercise in vivo. We analyzed both the cumulative BLI intensity for 4 h after TokeOni administration and the BLI intensity at each shooting time point over 4 h. The cumulative BLI intensity did not increase from 0 to 4 h after exercise, regardless of the duration of the exercise, but a single bout or 2 weeks of exercise enhanced the cumulative intensity of the BLI from 4 to 8 h after exercise. This result in the cumulative BLI intensity suggests that exercise stimuli enhanced total BDNF expression during 4–8 h after the end of exercise. The temporal expression revealed by BLI is consistent with the results of previous studies based on biochemical analyses [10]. In contrast, our previous BLI study showed that luminescence in the brain was upregulated significantly 1–3 h after a single boult exercise and tended to be upregulated within 24 h [15]. However, in a previous

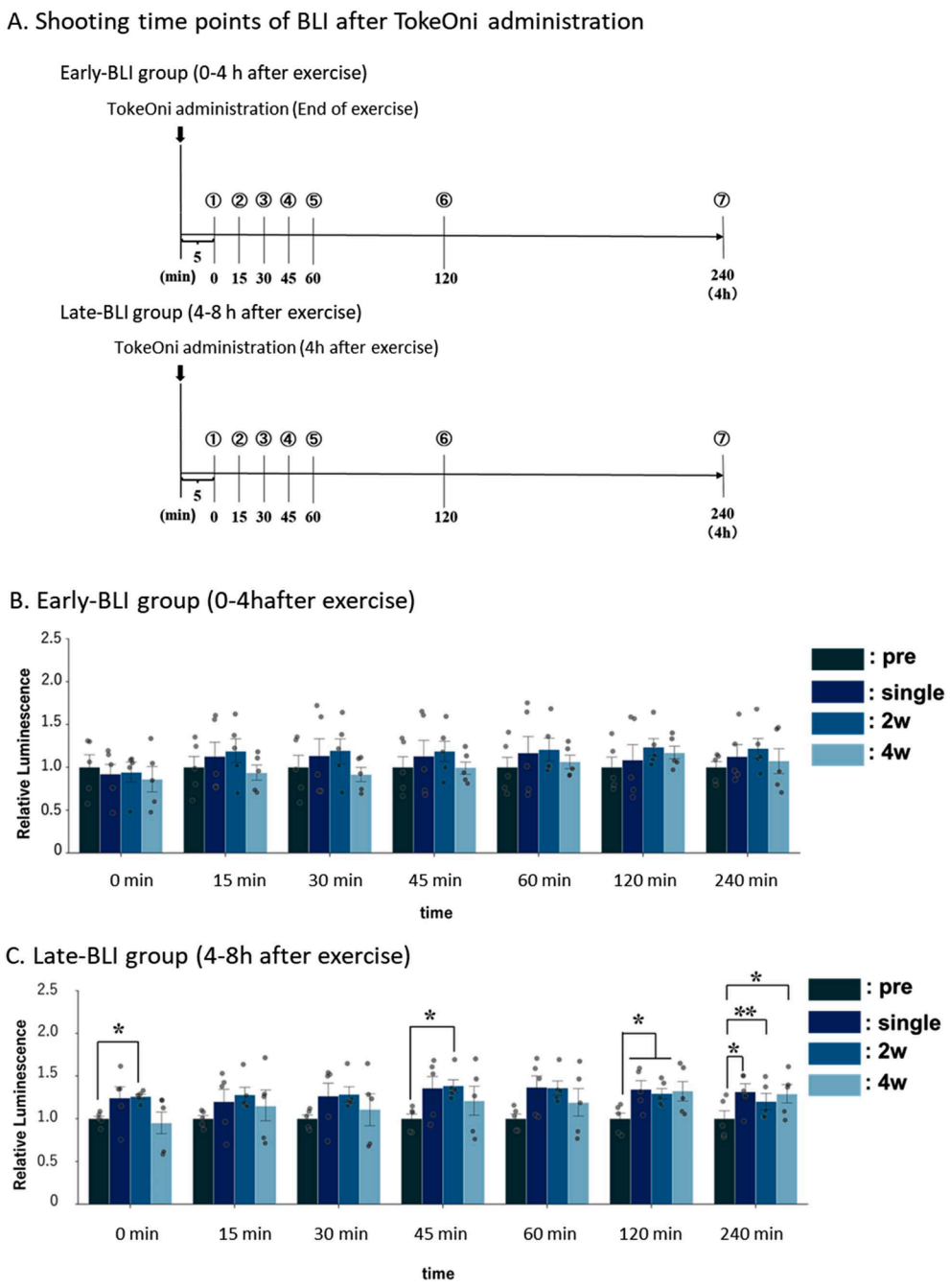

Fig. 3. Effect of exercise on the time-point bioluminescence level at each measurement timing. (A) Shooting time points of BLI after TokiOni administration. (B) Early-BLI group: BLI is performed approximately within 0–4 h after exercise. (C) Late-BLI group: BLI is performed approximately within 4–8 h after exercise. * or ** indicates a significant difference regarding exercise terms compared to pre in the post hoc test. ** Indicates $p < 0.01$. * Indicates $p < 0.05$.

study, a single bout of exercise with much higher intensity (15 m/min) was performed in older mice (6–7 months old) compared to the exercise regimen and the age of the mice used in this study (10 m/min and 8–9 weeks old). Therefore, the gap between the BLI studies suggests that the time window of BDNF expression could be modulated by factors related to the exercise regimen and characteristics of individual mice (including age, neurological and physical conditions referring to exercise load, and exercise habits), and that a higher level of exercise intensity could induce acute upregulation of BDNF expression after a single bout of exercise. Previous studies indicated that a high-intensity bout of exercise robustly increases BDNF expression in the brain [6]. Repeated high-intensity exercise, specifically at higher than the lactate threshold (supra-LT), negatively affects BDNF expression [21,25], indicating that the appropriate intensity for BDNF expression is controversial considering repeated exercise regimens.

Furthermore, 4–8 h after the end of exercise, a single bout of exercise after 2 weeks of exercise also significantly enhanced BDNF expression, whereas that after 4 weeks of exercise did not. This suggests that BDNF expression after a single bout of exercise could be altered depending on the frequency of repeated exercise, as reported in previous biochemical analyses targeting the hippocampus [17].

At each shooting time point, the enhancement of BDNF expression was first detected at 120 min in the late-BLI group, corresponding to 6 h after the end of exercise (Fig. 3B). After 2 weeks of exercise, an increase in BDNF expression was detected at 0 min in the late-BLI group, corresponding to 4 h after the end of exercise, suggesting that the

enhancement of BDNF expression triggered by a single bout of exercise was accelerated following repeated exercise for 2 weeks. However, after 4 weeks of exercise, the enhancement was delayed to 240 min in the late-BLI group, which corresponds to 8 h after the end of the exercise (Fig. 3B). Taken together, 2 weeks of exercise accelerated the onset of exercise-enhanced expression, while 4 weeks of exercise prevented this acceleration, indicating that repeated exercise could modulate the onset of exercise-enhanced BDNF in the brain. Similarly, the enhanced expression of total BDNF in cumulative BLI after 2 weeks of exercise was not detected after 4 weeks of exercise (Fig. 2C).

Previous studies based on biochemical analyses have shown that repeated exercise (exercise habits) can enhance exercise-dependent BDNF expression in the hippocampus and cortex [17–19]. This study is the first to show an accelerated onset of exercise-enhanced BDNF after a single bout of exercise. Considering the negative enhancement after 4 weeks of exercise, adaptive neuronal activity could be related. BDNF expression in the brain is modulated by neural activity in response to exercise stimuli [16,21]. A previous study examining the relationship between the duration of exercise and the expression of neural activity markers (c-fos) showed that c-fos expression peaks at approximately 1 week after the start of exercise and decreases over the subsequent 4 weeks [26]. It has also been reported that the acute enhancement of BDNF expression by the forced running exercise intervention is weakened by 4 weeks of voluntary exercise [9]. Furthermore, as a mechanism related to modulating BDNF expression, repeated exercise can modulate epigenetic regulation, altering the readiness of *BDNF* gene transcription [27]. Specifically, exercise acetylates histones at the BDNF promotor and conditions chromatin structures that are sensitive to gene transcription signals [28,29]. Moreover, recent studies demonstrated that exercise-induced metabolic factors in the peripheral organs, such as myokines, hepatokines, and ketones, pass through the blood–brain barrier and trigger BDNF expression in the brain [30]. Thus, it is reasonable to consider that repeated exercise can change the transcriptional readiness and metabolic reactions to a single bout of exercise.

In this study, an exercise regimen consisting of consistent intensity and duration was repeated for 4 weeks. Therefore, repeated exercise over 4 weeks could be suggested to induce a kind of neuronal and physical adaptation to the consistent level of exercise intensity and terms, reducing readiness for gene expression and sensitivity to the relative stimulus response to a single bout of exercise. Instead, it may be necessary to set an exercise regimen with progressively increasing intensity and duration of daily exercise to minimize the brain's adaptation to exercise when expecting exercise-enhanced BDNF expression in the brain to be upregulated by repeated exercise regimens.

In limitations, first, the BLI used herein reflected luminescence in the brain; however, it was difficult to reflect each specific brain region separately. Second, we did not elucidate the underlying biochemical mechanisms. This study revealed the time course of BDNF expression over repeated exercise, but it did not elucidate the biochemical mechanism underlying the modification of the time course.

5. Conclusion

Using BLI, this study showed that exercise enhances BDNF expression in the brain in vivo. BDNF expression was enhanced by a single bout of exercise, and this enhancement appeared 4–8 h after the end of exercise. Furthermore, exercise-dependent enhancement of BDNF expression was accelerated after 2 weeks of repeated exercise, but not after 4 weeks of repeated exercise. These outcomes were first demonstrated using in vivo BLI, a powerful tool to precisely verify the time course of BDNF expression over repeated exercise.

6. Significance of impacts

In vivo bioluminescence imaging (BLI) revealed that the start of BDNF expression enhanced by a single bout of exercise was accelerated after 2 weeks of repeated exercise, but not after 4 weeks of repeated exercise, indicating that repeated exercise modulates the time window of BDNF expression, which were first demonstrated using in vivo BLI, a powerful tool to precisely verify the time course of BDNF expression over repeated exercise.

CRediT authorship contribution statement

Ryo Ikegami: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Takahiro Inoue:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Yasuyuki Takamatsu:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Taichi Nishio:** Investigation. **Mamoru Fukuchi:** Writing – review & editing, Resources, Methodology. **Sanae Haga:** Writing – review & editing, Resources. **Michitaka Ozaki:** Writing – review & editing, Resources. **Hiroshi Maejima:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Funding acquisition, Conceptualization.

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

Data will be made available on request.

Acknowledgments

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