Abstract  Both exercise training and chronic caloric restriction contribute to brain health through enhanced expression of brain-derived neurotrophic factor (BDNF). This study investigated the synergistic effects between 12-week low-intensity exercise training and caloric restriction on hippocampal BDNF expression with redox status in rats. Twenty-six, 7-week-old male Wistar rats were randomly divided into the following 4 groups: (1) sedentary control (Con, n = 7), (2) exercise (Ex, n = 6), (3) caloric restriction (CR, n = 7), and (4) caloric restriction and exercise training (ExCR, n = 6). Although Con and Ex rats were fed ad libitum over time, CR and ExCR rats consumed 40% less food compared to Con rats. Ex and ExCR rats underwent low-intensity treadmill running (30 min/day, 5 days/week). Forty-eight hours after the termination of the 12-week intervention, rats were sacrificed and the hippocampus was quickly dissected for measuring BDNF expression and markers of oxidative stress, including 4-hydroxy-2-nonenal (4-HNE). Hippocampal BDNF expression was significantly increased in Ex compared to Con rats (p = 0.007), whereas the exercise-induced increase in BDNF was completely suppressed by a combination with caloric restriction. Furthermore, we observed a significant relationship between hippocampal BDNF and 4-HNE expression (r = 0.725, p < 0.001). Our findings indicate that exercise training combined with caloric restriction might not have a synergistic effect on hippocampal BDNF expression in young rats. Moreover, exercise-induced oxidative stress can trigger BDNF expression in the hippocampus.

Keywords: exercise, diet, combined effect, brain-derived neurotrophic factor, 4-hydroxy-2-nonenal

Introduction  In our aging society, long-term maintenance of brain function is a public health priority. For many people, cognitive decline is a major facet of the ageing process. This is linked to unhealthy lifestyle behaviors, such as physical inactivity and excessive caloric intake. Regular exercise is known to have beneficial effects on cognitive function, including attention, memory and working memory, learning, and judgement. One important molecular mediator of exercise-induced benefits to the brain is the presence of neurotrophic factors, of which brain-derived neurotrophic factor (BDNF) is the most notable. Neurotrophic factors are pivotal for many central nervous system functions such as neuronal survival, migration, and synaptogenesis. In particular, BDNF plays crucial roles in the brain development, maintenance, and plasticity of neurons, which contribute substantially to the prevention of depression and Alzheimer diseases through maintaining or improving memory and learn-
It is widely accepted that exercise training stimulates various signaling mechanisms that lead to BDNF upregulation, particularly in the hippocampus, thereby modulating cognitive functions by improving neurogenesis and neuronal activity. Previous studies demonstrated that low-intensity exercise increased hippocampal BDNF and gene expression to a greater extent than moderate- or vigorous-intensity exercise.

It has been demonstrated that caloric restriction extends lifespan by delaying the onset of age-related diseases, including brain dysfunction. Caloric restriction is a dietary intervention that reduces 20-40% of the energy from normal ad libitum food intake (AL). A previous study suggested that a 12-week intervention of 40% caloric restriction significantly increased hippocampal BDNF expression compared to AL. In addition, there is growing evidence that hippocampal BDNF expression is elevated by regular exercise or caloric restriction alone. The combined effects of exercise and dietary intervention on BDNF expression have also been investigated. Stranahan et al. demonstrated that 12-week combined intervention of voluntary wheel running and 40% caloric restriction increased hippocampal BDNF expression compared to voluntary exercise and caloric restriction alone in young wild-type mice. They suggested that the additive effects of the combination of exercise and diet on BDNF expression were due to increased energy metabolism in the periphery. Khabour et al. assessed the effects of every other day fasting and voluntary wheel running for 6 weeks in young rats, and the combined intervention significantly increased BDNF expression, as well as improved cognitive performance evaluated by the radial arm water maze. However, the underlying mechanism of the synergistic effect of exercise and caloric restriction on these hippocampal benefits remains to be elucidated.

We hypothesized that “oxidative stress” would be one of the possible triggers for exercise- and/or caloric restriction-induced hippocampal BDNF expression. Oxidative stress is defined as “a disturbance in the oxidants and antioxidants balance in favor of the oxidants”. Although moderate levels of oxidative stress generation are associated with positive adaptive responses (e.g., neurogenesis and plasticity), high levels result in brain inflammation and dysfunction. Indeed, eight weeks of swimming exercise increased both cortical malondialdehyde (MDA) levels and BDNF mRNA expression, while vitamin E ingestion attenuated exercise-induced BDNF mRNA expression. In contrast, vigorous exercise decreased BDNF contents with increasing oxidative damage in mice brain. Hence, these studies raise the possibility that the levels of oxidative stress can affect BDNF expression in the hippocampus, indicating that synergistic adaptive responses of exercise and caloric restriction might be changed by redox status. However, previous studies did not examine the relationship between hippocampal BDNF contents and oxidative stress through the combined intervention of chronic exercise and caloric restriction. Therefore, we investigated the synergistic effect of low-intensity exercise training and caloric restriction on hippocampal BDNF levels in view of redox status in rodents.

Methods

Animals. All animal experiments were conducted in accordance with the experimental animal guidelines published by the Japanese Ministry of Education, Culture, Sports, Science, and Technology and approved by the University of Yamanashi Animal Care and Use Committee (no. 19-90).

The experiments were carried out with twenty-six 7-week-old Wistar male rats (SLC, Tokyo, Japan). The animals were housed 1 per cage under 12 h light/dark cycle at 22 °C with free access to water. Prior to the intervention, the animals were allowed to acclimatize for 1 week in order to become familiar with low-intensity running exercise (10 m/min, 10 min/day) using a rodent-specific treadmill (TM-N1-V100, Osaka Micro Systems, Osaka, Japan).

Experimental intervention. After the acclimatization period, the animals were weight matched (190-220 g) and divided into the following 4 groups: (1) sedentary control (Con, n = 7), (2) exercise (Ex, n = 6), (3) caloric restriction (CR, n = 7), and (4) exercise training and caloric restriction (ExCR, n = 6). Subsequently, animals were raised under the lifestyle intervention of exercise and diet until 12 weeks (from 8 weeks to 20 weeks old). Con and Ex rats were fed standard chow (MF, Oriental Yeast Co. Ltd., Tokyo, Japan) ad libitum throughout the intervention. Every day, food intake of Con and Ex was measured, and subsequently the same chow was fed to CR and ExCR rats at a quantity of 40% less compared to Con rats. Ex and ExCR rats underwent 30-min treadmill running (5 ° slope, 10 m/min, 50-55%VO2 max). Non-exercise groups (Con, CR) were kept on the treadmill for the same duration (30 min) to eliminate the effect of handling stress on differences in physiological responses between the four groups. The training on a motorized treadmill was conducted five times per week for 12 weeks. For all rats, body weight was weighed daily during the 12-week intervention. The animals were starved for 48 h after the last training session (at 12-week intervention) and sacrificed by extracting the blood from the celiac artery under anesthesia (with diethyl ether until loss of consciousness, 2-3 min). The brain of each animal was quickly removed and stored at -80°C for further analysis.

Biochemical analysis. As an index of lipid peroxidation, 4-hydroxy-2-nonenal (4-HNE) levels in the hippocampus were measured by enzyme-linked immunosorbent assay (ELISA) analysis. The hippocampus was homogenized
in buffer (phosphate buffered saline: PBS containing 1% protease inhibitor) at 15 μl per 1 mg of hippocampus. 4-HNE protein levels were measured using the OxiSelect™ HNE-His Adduct ELISA Kit (STA-334, Cell Biosciences, Inc., San Diego, USA) according to manufacturer protocol. In addition, total antioxidant capacity (TAC) in the hippocampus was measured using the potential antioxidant (PAO) assay kit (KPA-050O, Japan Institute for the control of Aging, Shizuoka, Japan). This assay is based on the reduction of Cu²⁺ to Cu⁺ by the mixed reaction of all the antioxidants in the hippocampal samples.

For quantitative detection of BDNF levels in the hippocampus, the dispensed homogenates were mixed with the same quantity of lysis reagent (ProteoJET™ Mammalian Cell Lysis Reagent, Fermentas, USA) and centrifuged at 17,800 x g for 3 min (4 °C). The supernatants were used for standard sandwich ELISA analysis using the Rat BDNF ELISA Kit (EK0308, Boster Biological Technology, Pleasanton, USA) according to the manufacturer’s instructions. Furthermore, protein concentration in the supernatants used for the 4-HNE and BDNF assays was also assessed. Protein concentration was determined by BCA protein assay (Thermo Fisher Scientific, Massachusetts, USA).

Data and statistical analysis. Data were expressed as mean ± standard deviation (SD). The statistical comparisons were made using two-way (exercise × diet) ANOVA with subsequent Tukey’s HSD test as post-hoc analysis. For comparison of the mean food intake of Con and Ex during 12 weeks, unpaired t-test was performed.

Moreover, Pearson’s correlation coefficient was used to examine the relationship between BDNF and redox status in the hippocampus, i.e., 4-HNE levels. All statistical analyses were performed using R ver. 3.1.2. A value of p < 0.05 was considered significant.

Results

Food intake and body weight gain. Mean food intake of AL rats (Con, Ex) during the 12-week intervention was 15.0 ± 0.6, 15.3 ± 0.5 g/day, respectively. No significant difference was detected with regard to food intake between Con and Ex (t = 0.917, p = 0.379).

Fig. 1 shows the mean percentage body weight gain for each group at the end of the 12-week intervention. No significant interaction was detected between exercise and diet with regard to body weight gain (F_{1,22} = 1.167, p = 0.292). However, a significant effect of the diet was detected (F_{1,22} = 572.345, p < 0.001) without exercise (F_{1,22} = 1.815, p = 0.192). Specifically, the rats assigned to the restricted diet groups (CR, ExCR) showed a smaller percentage of body weight gain of approximately 55% compared to the AL groups (Con, Ex).

Hippocampal redox status. Fig. 2 shows the hippocampal redox status evaluated by 4-HNE levels (Fig. 2A) and TAC (Fig. 2B) after the 12-week intervention. A significant interaction was detected with regard to the effects of
exercise and diet on hippocampal 4-HNE levels ($F_{1,22} = 37.981$, $p < 0.001$, Fig. 2A). 4-HNE levels in Ex and CR rats were significantly greater than in Con rats ($p < 0.001$). In contrast, 4-HNE levels in ExCR rats were significantly lower than in Ex and CR rats (Ex: $p < 0.001$, CR: $p = 0.021$, respectively). In TAC, no significant interaction was detected between exercise and diet, and no main effects were identified (exercise: $p = 0.866$; diet: $p = 0.691$; interaction: $p = 0.781$, Fig. 2B).

Hippocampal BDNF expression. Fig. 3 illustrates hippocampal BDNF levels after the 12-week intervention. A significant interaction was detected between exercise and diet with regard to the BDNF levels in the hippocampus ($F_{1,22} = 9.400$, $p = 0.006$). Hippocampal BDNF contents in Ex rats were significantly greater than in Con rats ($p = 0.007$). However, those in ExCR rats were significantly lower than in Ex rats ($p = 0.003$). Moreover, hippocampal BDNF expression significantly correlated with 4-HNE levels ($r = 0.725$, $p < 0.001$, Fig. 4).

Discussion

We examined the synergistic effects between 12-week low-intensity exercise training and caloric restriction on hippocampal BDNF expression in view of redox status in young male rats. Our findings demonstrate that low-intensity exercise training significantly increases both BDNF and 4-HNE levels in the hippocampus. However, exercise training conducted simultaneously with caloric restriction completely suppressed the exercise-induced elevation in both hippocampal BDNF and 4-HNE levels. Moreover, BDNF expression significantly correlated with 4-HNE levels in the hippocampus. These results indicate that oxidative stress induced by low-intensity exercise training might contribute to BDNF expression in the hippocampus. In contrast, the attenuated increase in oxidative stress by exercise training combined with caloric restriction has a negative effect on exercise-induced BDNF expression.

Previous studies demonstrated that 40% of caloric restriction increased BDNF contents in the hippocampus18,19). Although severe (over 50%) caloric restriction increased lipid peroxidation, moderate (30-40%) caloric restriction increased antioxidant capacity without oxidative damage in the liver20). Because of these previous results, we expected that 40% caloric restriction would be an effective and secure intervention to elevate hippocampal BDNF expression. According to the manufacturer’s data (SLC, Tokyo, Japan), rats during the 12-week intervention (from 8 weeks to 20 weeks old) were in a growth period. However, the body weight of the restricted diet group (CR, ExCR) increased by just 15%. In contrast, that of the AL group (Con, Ex) increased by 75% (Fig. 1). In this study, we intervened by caloric restriction for young rats, because the late-onset of caloric restriction in old mice did not ameliorate the working memory27). Nevertheless, it seems that 40% of caloric restriction for young rats in our study might be more severe than we had expected.

Several studies suggest that the status of oxidative stress regulated by reactive oxygen species (ROS) generation may affect BDNF expression23,24,28-32). There are many antioxidant markers, but it remains unknown which antioxidant marker is applicable to reflect antioxidant status relating to BDNF levels in the hippocampus. TAC, assessed by the kit we used, can detect the capacities of both water- and fat-soluble antioxidants; we thus considered TAC as the most suitable biomarker to evaluate the overall antioxidant status in the hippocampus. However, there were no significant differences in TAC among the four experimental groups (Fig. 2B). A previous study demonstrated that a 12-week combination of moderate exercise training and 30% caloric restriction elevated the reduced glutathione (GSH) levels, but did not change superoxide dismutase (SOD) activity in rat hippocampus33). On the other hand, mild exercise training decreased GSH, SOD, and catalase activities in cerebral cortex23). Furthermore, mild levels of caloric restriction increased GSH and SOD activities, but more severe levels decreased or cancelled such up-regulation26). Therefore, we speculate
the possibility that exercise training and caloric restriction might promote both an elevation and reduction in various antioxidant capacities. This may explain why significant differences of TAC among the four groups were hardly detected.

We have previously reported that antioxidant administration to attenuate high-intensity exercise-induced hippocampal 4-HNE overproduction results in enhanced BDNF expression in the rat hippocampus. For this reason, we assessed hippocampal 4-HNE levels with BDNF in this study. As a result, low-intensity exercise training significantly increased hippocampal 4-HNE levels (Fig. 2A). Moreover, there was a strong correlation between 4-HNE and BDNF levels in the hippocampus (Fig. 4). Previous studies demonstrated that exercise-induced BDNF production significantly correlated with ROS generation. Furthermore, Sakr et al. reported antioxidant ingestion attenuates moderate exercise training-elevated BDNF gene expression in rat brain. Our present findings are in accordance with these previous results. Taken together, the production of ROS, evaluated as increased 4-HNE, might be important for increasing BDNF levels in the hippocampus. On the other hand, oxidative stress by ROS generation can induce “hormesis”, which is defined as a situation in which low-dose stimulation induces various positive physiological responses; but high-dose stimulation has harmful consequences. It is suggested that intense exercise increased lipid peroxidation and decreased BDNF levels in mice brain. In addition, we have observed antioxidant ingestion-augmented hippocampal BDNF expression via decreasing high-intensity exercise-induced 4-HNE levels. Therefore, we support the hypothesis that low-intensity exercise-induced “moderate” oxidative stress can trigger hippocampal BDNF expression.

Interestingly, the elevation in the hippocampal BDNF levels was not detected in CR and ExCR (Fig. 3). The reason for this remains unclear. However, if there was a possible explanation, we would speculate that it might be related to the level of corticosterone (CORT). CORT is known to attenuate 4-HNE production in the brain, and its overproduction impairs hippocampal BDNF expression and neurogenesis. A previous study suggested that the oxidative damage increased as the severity of dietary restriction was elevated, and 40% (relatively severe) caloric restriction could increase the 4-HNE production in the hippocampus. In contrast, over 30% caloric restriction can also elevate CORT levels. From these observations, we assume that the increase in 4-HNE levels of CR and ExCR might be attenuated by elevating CORT. Furthermore, acute mild (15 m/min) treadmill exercise did not affect CORT induction, whereas chronic exercise at the same load accumulated serum CORT levels. Accordingly, treadmill exercise training could enhance the CORT production induced by 40% caloric restriction, which might lead to greater suppression of the increase in hippocampal 4-HNE levels in ExCR rats. We confirmed the 4-HNE levels were significantly associated with BDNF expression in the hippocampus. Therefore, the attenuated increase in 4-HNE levels might be insufficient to facilitate the BDNF expression in CR and ExCR rats. However, these explanations are highly speculative, as CORT levels were not assessed in the present study.

The present study has at least two limitations. First, we could not evaluate how BDNF induction by the 12-week intervention contributes to the improvement of cognitive function assessed by behavioral tests. However, increased BDNF expression was associated with improved performance on hippocampal-related cognitive tasks such as learning and memory, indicating that increased BDNF due to low-intensity exercise training may improve cognitive performance in this study. Second, although we showed a correlation efficient between oxidative stress and BDNF expression in the hippocampus, we did not assess the pathway connecting them. It is suggested that ROS generated by moderate exercise increased BDNF via nuclear factor kappa B (NF-κB) and cAMP response element binding protein (CREB) pathways. Taken together, future studies should investigate these indexes.

In summary, low-intensity exercise training combined with 40% caloric restriction might not have a synergistic effect on BDNF expression, due to the suppression of the increase in hippocampal 4-HNE content in young male rats. Our findings suggest that severe caloric restriction suppresses exercise-induced beneficial brain adaptation in younger subjects. Moreover, 4-HNE may play an important role in low-intensity exercise-induced BDNF expression in the hippocampus.

**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of this article.

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**References**

4. Wrann CD, White JP, Salogiannnis J, Laznik-Bogoslavski D,


