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Continuous environmental enrichment and aerobic exercise improves spatial memory: focus on rat hippocampal BDNF and NGF

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RESEARCH ARTICLE

Abstract

Memory is an important cognitive function in humans. Exercise and environmental enrichment (EE) exposure have positive effects on memory function via improved neurogenesis through expression of growth factors such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF). Exercise and environmental enrichment have independently been shown to increase BDNF and NGF, but the effect of the combination of these treatments has not been widely studied. This experimental study aims to analyse the effect of aerobic exercise, EE exposure, and combination of aerobic exercise and EE exposure on memory function. This study used twenty 7-month old male Wistar rats that were given treatment for 8 weeks. Memory function was tested using forced alternation Y-maze. Hippocampal expression of BDNF and NGF were also assessed. The results showed the combination group has highest performance in memory function test and also the highest level of hippocampal BDNF and NGF (P<0.05). It can be concluded that the combination of aerobic exercise and continuous EE exposure produces the best results for memory function through higher levels of hippocampal BDNF and NGF in adult rats.

Keywords: environmental enrichment, aerobic exercise, BDNF, NGF, spatial memory

1. Introduction

The ability to store and recall information is one of the most amazing abilities of higher organism (Cunha *et al.*, 2010). Memory is fundamental to the understanding about oneself and the world, thus, memory loss is destructive for an individual's quality of life (Warburton, 2015). Memory decreases with age as a degenerative process, and in some cases might even lead to pathological memory impairment (Kovacs, 2015). The formation and stabilisation of synapse contacts is the final stage in neural development and the process of forming memory function. This process occurs in the hippocampus (Vivar *et al.*, 2013). Due to the importance of memory in human life, many studies looked into the process of memory formation by looking in changes in the structure and function of the hippocampus. One of the emerging studies today is the study of molecular mechanisms of memory formation associated with synaptic plasticity by measuring levels of memory-related proteins located in synapses, as well as looking at expression of neurotrophic factors in cases of memory impairment and the inability to process and store new memory (Vivar *et al.*, 2013). Until today, there is no curative treatment for the declining of memory function, thus preventive efforts should be implemented by increasing synaptic plasticity as early as possible in young adulthood. In addition, behavioural manipulation can also prevent memory decline, such as physical exercise and environmental enrichment (EE) (Orlandi *et al.*, 2007).

Physical exercise is classified into two types, aerobic and anaerobic. Various studies have shown that physical exercise is able to alter the structure and function of the brain. Aerobic exercise increases blood flow to the brain including hippocampus by angiogenesis (Grif *et al.*, 2011). Aerobic exercise has also been shown to increase the activity of nerve cell signals that induce LTP, which is known through increased neurotrophic factors expression in the

hippocampus, including insulin-like growth factor 1 (IGF-1), fibroblast growth factor-2, brain-derived neurotrophic factor (BDNF), and nerve growth factor (NGF) (Maass *et al.*, 2016). The neurotrophic factor that were increased after induced by exercise is BDNF. A recent study by Huan *et al.* (2013) demonstrates strong expression of BDNF post-exercise in different parts of the human brain including the hippocampus, claustrum, and amygdala. It has been shown that BDNF plays an important role in the development of synapse and its plasticity (Huan *et al.*, 2013).

In a separate treatment it has been shown that both aerobic exercise and EE exposure have positive effect on memory function (Constans *et al.*, 2016; Ickes *et al.*, 2000). Aerobic exercise has been shown to increase blood flow to the brain, permeability of blood brain barrier, and expression of growth factors like BDNF and NGF. EE has been shown to improve memory function through increased activity of cholinergic neurons that could promote NGF regulated pathway (Torasdotter *et al.*, 1998). Moreover, EE could overexpress BDNF through increased motor activity provided in EE model (Mosaferi *et al.*, 2015). Even though exercise and environmental enrichment has independently been shown to increase BDNF and NGF, but the effect of the combination of these treatments has not been widely studied. Therefore, in this study we combined aerobic exercise and EE to improve memory function with focus on the expression of hippocampal BDNF and NGF.

2. Material and methods

Animals

An *in vivo* experimental study on 20 adult male Wistar rats age seven months, with weight ranging from 306 to 361 g. Rats were acquired from Rat Lab Indo (Jakarta, Indonesia). The rats were randomly assigned to four groups: Control (C) (n=5), Aerobic Exercise (A) (n=5), Environmental Enrichment (EE) (n=5), and combination of Environmental Enrichment and Aerobic Exercise (EEA) (n=5). Rats without EE treatment were housed in pairs, two rats in each cage. They were maintained under a 12 h light/dark cycle with food and water available *ad libitum*. The food given was standard AIN-93M diet (TestDiet, St. Louis, MO, USA). Rats were acclimatised two weeks before the start of the experiment. In the first acclimatisation week, rats are placed in the experiment room and handled by researchers for familiarisation. In the second acclimatisation week, groups treated with aerobic exercise were introduced to the treadmill with increasing speed up to 15 m/min for no more than 10 min each day, while groups treated with EE were placed at the Marlau™ cage for 15 min each day, and at the end of the week all rats were introduced to the Y-maze for 3 min. At the end of an 8 week experiment period, rats were sacrificed by cervical dislocation, and hippocampus was isolated (Spijker, 2011) (Supplementary materials and methods S1). Rat ELISA kits were used to measure hippocampal BDNF (QY-E11168) and NGF (QY-E11105) levels (Qayee-bio, Shanghai, China P.R.). The design and method of this research have been approved by the Health Research Ethics Committee, Faculty of Medicine, Universitas Indonesia no.1018/UN2.F1/ETIK/2017.

Aerobic exercise

Aerobic exercise was given to group A and EEA using a four-lane treadmill for rats. The treatment was given for 8 weeks, 5 days/week, with each session lasting for 30 min with treadmill speed of 20 m/min. Before each session, there was a 3 min warming up session with eight m/min treadmill speed. To confirm that the exercise treatment given is indeed aerobic exercise, a preliminary study was performed on five rats in which their lactate plasma levels were assessed directly at the end of the exercise session, and all five rats had lactate plasma levels below 4 mmol/l with average of 3.18±0.26 mmol/l.

Environmental enrichment model (Marlau cage)

The EE model used was a standardised apparatus, the Marlau cage (Figure 1; Fares, 2009; Sanchez *et al.*, 2009). The goal of a 24 h continuous EE exposure is to improve the quality of life of the animals by providing a combination of physical activity, enhanced social interaction, and natural exploration (Mora *et al.*, 2007). The EE model was given for group EE and EEA. The treatment was given for 8 weeks (Abdullah, 2017). The Marlau cage size is 800×600×510 mm, consisting of two floors and has various enrichment objects such as running wheels, ladders, labyrinths, plastic houses, tunnels, and nesting materials. The enrichment objects used are in bright colours to provide visual sensory stimulation. Ground floor height is 300 mm with G1 area measuring 296×600 mm and G2 area measuring 496×600 mm. Then, there is a labyrinth on the upper floor, connected from G2 area by a stair and there is also a sled tunnel going to G1 area.

According to the protocol, the labyrinth configuration was changed three times per week, on Mondays, Wednesdays, and Fridays using six labyrinths (A-F), each consisting of two different configurations (1 and 2). The maze change begins with the A1 series, B1, C1 and so forth until it ended with D2, E2, and F2. When the maze change ended with the F2 series it starts back from the A1 series (Table 1).

Forced alternation Y-maze

Memory function task was performed in all groups using a Y-maze (Figure 2; Wolf *et al.*, 2016). The Y-maze used was a symmetrical Y-maze made of black wood veneer for the bottom plate and walls. Each arm of the Y-maze is 40 cm long, 8 cm wide, and 15 cm high. To reduce animals' anxiety, the light level in the test room was reduced to 30±5 lux (Wolf *et al.*, 2016).

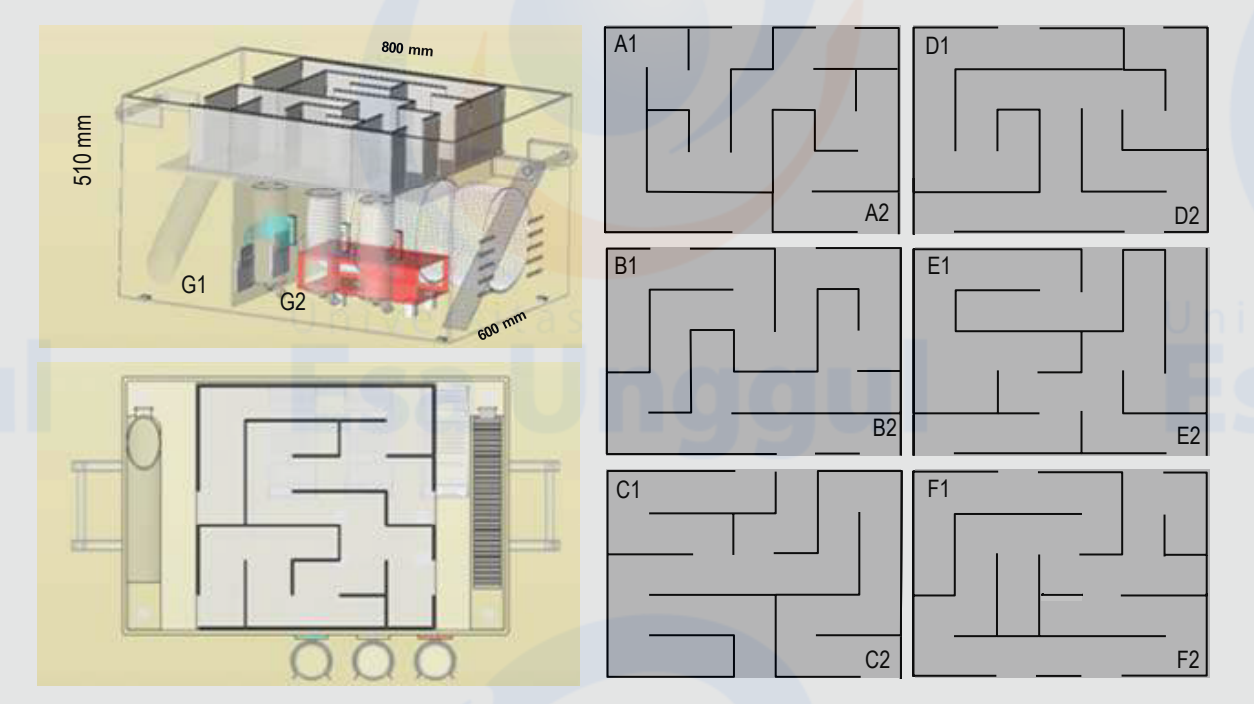

Figure 1. Marlau™ cage (Fares, 2009; Sanchez *et al.*, 2009).

Table 1. Configuration changes of Marlau cage maze (Abdullah, 2017; Dita, 2017).

Marlau	Day	Maze series
Week 1	Monday	A1
	Wednesday	B1
	Friday	C1
Week 2	Monday	D1
	Wednesday	E1
	Friday	F1
Week 3	Monday	A2
	Wednesday	B2
	Friday	C2
Week 4	Monday	D2
	Wednesday	E2
	Friday	F2
Week 5	Monday	A1
	Wednesday	B1
	Friday	C1
Week 6	Monday	D1
	Wednesday	E1
	Friday	F1
Week 7	Monday	A2
	Wednesday	B2
	Friday	C2
Week 8	Monday	D2
	Wednesday	E2
	Friday	F2

Figure 2. Y-maze.

The memory function test used with the Y-maze was the forced alternation test. The protocol for the forced alternation test was modified from Melnikova *et al.* (2006). Starting three days before the test, rats were handled. The test was carried out during the dark period and consisted of a 5 min sample trial (T1) followed by a 5 min retrieval trial (T2). In T1, the rat was placed facing the wall and away from the centre at the end of the start arm. Then, the rat was permitted to explore two Y-maze arms, while the third arm was blocked to prohibit entry to the arm. After T1, the

rat was returned to its home cage for 30 min, the intertrial interval. For T2, the third arm was freely explorable as the block was removed. The rat was placed at the start arm again, and then the rat was permitted to explore all three arms of the Y-maze (Wolf *et al.*, 2016). If a rat climbed above the wall of the maze, it was returned immediately into the respective arm of the maze. The parameter measured in this memory test was time in novel arm. The definition of time in novel arm (%) is time spent in the novel arm divided by time spent in all arms during the T2 retrieval trial. Rats with low mobility, defined as entering less than three arms in the first min of T2 were excluded from analysis (Wolf *et al.*, 2016).

Brain-derived neurotrophic factor and NGF levels

Whole hippocampus tissue was isolated (Supplementary Materials and methods S1) and then homogenised with phosphate buffer saline pH 7.4 and was centrifuged for 10 min at the speed of 3,000 rpm, and then the supernatant was collected and stored at -80 °C until further processing. Total protein concentration was measured with Bradford protein assay then compared to BDNF and NGF concentration that has been measured with ELISA kit, thus the hippocampal BDNF and NGF levels are reported in pg/mg protein. Hippocampus samples were assessed for BDNF and NGF levels with a commercially available rat ELISA kit from Qayee-Bio.

Statistical analyses

The BDNF and NGF levels were analysed using one-way ANOVA test, while the memory function was analysed using two-way ANOVA with repeated measures, with Bonferroni as the post-hoc test. The level of statistically significant value was set to $P<0.05$. Statistical tests were carried out using SPSS (IBM, Armonk, NY, USA).

3. Results

Body weight, baseline locomotor activity, protein levels

All groups had no significant difference in body weight at the start of the experiment. At the end of the experiment, the combination group had the lowest body weight compared to other groups and was significantly different from the control group ($P=0.048$; Table 2). All groups spent similar amount of time in all arms of the Y-maze at the start of the experiment, while at the end of the experiment the EE group spent significantly more time in all Y-maze arms than the control group ($P=0.013$), and the combination group also had the highest time spent in all Y-maze arms compared to other groups with significant difference with control group ($P=0.002$). Total hippocampal protein levels also were not significantly different between groups.

Forced alternation Y-maze

Alternation task measures rats' natural behaviour to explore new environments and is used to assess memory and exploratory behaviour (Figure 3). Results showed the percent time spent in the novel arm for control, aerobic, EE, and EEA groups.

Hippocampal brain-derived neurotrophic factor

The combination of continuous EE exposure and aerobic exercise induced the highest levels of hippocampal BDNF concentration (Figure 4). The hippocampal BDNF level in control (C) group was the lowest (34.30±5.65), followed by aerobic exercise (A) group (35.96±5.67), environmental enrichment (EE) group (39.95±6.48), and combination (EEA) group (47.16±5.62). There were significant differences between EEA and C group ($P=0.008$), and between EEA and A group ($P=0.04$).

Table 2. Comparison of body weight, baseline locomotor activity, and total protein levels.[1]

Parameter	Group C	Group A	Group EE	Group EEA	*P*-value
Body weight week 0 (g)	324±18.1	345±16.6	339.6±18.23	337.6±19.05	>0.05
Body weight week 8 (g)	397.8±11.76	384.6±9.15	389±8.25	379.6±8.44	0.048 EEA vs C
Total time in all arms week 0 (s)	229.2±20.29	175.4±33.92	209±38.11	188.6±6.66	>0.05
Total time in all arms week 8 (s)	153.6±45.14	166.6±24.42	222.8±29.94	241.6±7.37	0.013 EE vs C 0.002 EEA vs C
Total hippocampal protein level (mg)	4.72±0.26	4.76±0.31	3.83±0.21	3.67±0.25	>0.05

[1] A = aerobic; C = control; EE = environmental enrichment; EEA = combination. Data presented as mean ± standard deviation.

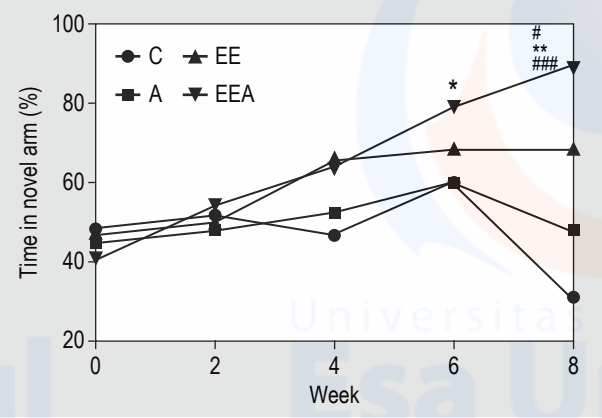

Figure 3. Comparison of percent time spent in novel arm between groups and treatment week. A = aerobic; C = control; EE = environmental enrichment; EEA = combination. * EEA week 6 vs EEA week 0 (*P*<0.05); ** EEA week 8 vs EEA week 0 (*P*<0.01); # EEA week 8 vs A week 8 (*P*<0.05); ### EEA week 8 vs C week 8 (*P*<0.001).

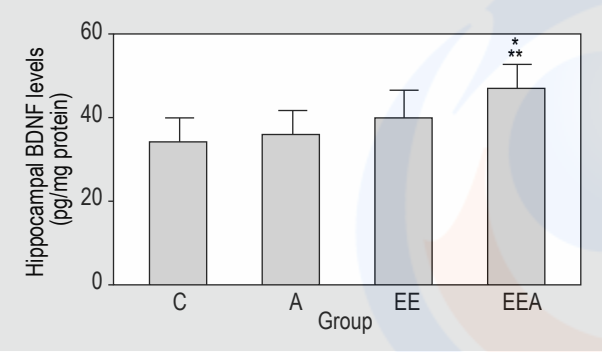

Figure 4. Mean hippocampal brain-derived neurotrophic factor (BDNF) levels. Error bars represent standard deviation. A = aerobic; C = control; EE = environmental enrichment; EEA = combination. *EEA vs A (*P*<0.05); **EEA vs C (*P*<0.01).

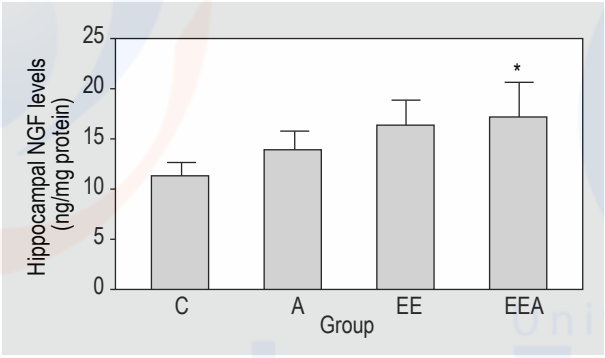

Figure 5. Mean hippocampal nerve growth factor (NGF) levels. Error bars represent standard deviation. A = aerobic; C = control; EE = environmental enrichment; EEA = combination. * EEA vs C (*P*<0.05).

Hippocampal nerve growth factor

The combination of continuous EE exposure and aerobic exercise induced the highest levels of hippocampal NGF concentration (Figure 5). The hippocampal NGF level in control (C) group was the lowest (11.17±1.37), followed by aerobic exercise (A) group (13.66±1.81), environmental enrichment (EE) group (15.80±3.50) and environmental enrichment and aerobic (EEA) group (16.91±3.59). There was a significant difference between EEA and C group (P=0.026).

4. Discussion

The rats were seven months old at the start of the experiment. This is comparable to twenty-one years old in humans, an age in which synapses have matured and stabilised, and memory decline starts to occur (Aine *et al.*, 2011). Treadmill speed of 20 m/min is classified as low-moderate intensity exercise, the best exercise intensity for improving memory function (Kartinah *et al.*, 2018). Treatment duration of eight weeks was chosen based on previous study results showing that our aerobic exercise regimen improves rats' neuroplasticity and spatial memory starting at week six (Kartinah *et al.*, 2018), thus the treatment duration was lengthened to observe whether an eight-week treatment duration would further enhance memory function. The results of our study were in line with this, in which memory function enhancement in combination group starts at week six, and further increases at week eight. An additional two weeks exercise regimen might be beneficial, this needs to be studied further. Rats' body weight at the start of the experiment ranged from 306-361 g. At seven months old, or approximately 30 weeks old, this value is lower than other reported studies (Nistiar *et al.*, 2012; Sengupta, 2013). Even though rats' body weight can sometimes be considered an indicator of its age, weight is not an accurate surrogate marker for age. Male rats of the same exact age showed up to 100 g variation in body weight (Sengupta, 2013).

Physical exercise and environmental enrichment can improve memory function through various pathways. Abdullah and Dita have demonstrated that aerobic exercise and EE have a positive effect on memory function (Abdullah, 2017; Dita, 2017). In line with this, the results of this study showed that combination of aerobic exercise and continuous environmental enrichment improves memory function the best. One of the major limitations of this study is that the cognitive outcomes were measured using only the Y-maze. We propose using other cognitive tests in future studies. Another possible cofounding factor that may affect the memory function test results is the lower body weight in the combination group at the end of the experiment, causing higher locomotor activity in general. This may be caused by the combination of increased opportunity of

voluntary physical activity in the enriched environment and the exercise regimen given to the combination group.

Exercise, especially aerobic exercise, can increase cognitive function related to increased growth factor expression such as BDNF, vascular endothelial growth factor, IGF-1, and NGF (Maass *et al.*, 2016). BDNF is a growth factor induced by physical exercise (Wrann *et al.*, 2013). A recent study showed that fibronectin type III domain-containing protein 5 (FNDC5) expression in the rats and mice hippocampus is increased in treatment of aerobic exercise (Wrann *et al.*, 2013). FNDC5 is a muscle protein that is induced in exercise and is cleaved and secreted as irisin. Administration of peripheral FNDC5 to the liver through adenoviral vectors results in increased irisin levels in the blood and induces expression of BDNF and other neuroprotective genes in hippocampus (Wrann *et al.*, 2013). The effects of exercise on the brain are most apparent in the dentate gyrus of hippocampus, the part of the brain that plays a role in learning and memory. Specific beneficial effects of exercise in the brain that have been reported includes increased size and blood flow to human hippocampus, dendritic and dendritic spine morphological changes, and increased synaptic plasticity (Cotman *et al.*, 2007).

Environmental enrichment provides a complex stimulation to the sensory and motoric system by offering animals opportunities for physical activity, various learning experiences and social interaction (Will *et al.*, 2004). The presence of a running wheel providing opportunity for voluntary physical exercise is an important component of an enriched environment (Van Praag *et al.*, 2000; Will *et al.*, 2004). Living in an enriched environment creates several plastic changes in the adult brain, especially in the hippocampus and cerebral cortex (Van Praag *et al.*, 2000). These plastic changes are supposed to reflect the reorganisation of neural connections that are involved in sensory-perceptual processing and learning. Probable candidates mediating these changes are neurotrophins such as BDNF, NGF, and neurotrophin-3 (NT-3) following environmental enrichment (Bekinschtein *et al.*, 2011; Torasdotter *et al.*, 1998). NGF has been linked with cognitive function and has been proven to improve the performance of cholinergic neurons (Torasdotter *et al.*, 1998).

BDNF and NGF are neurotrophic factors which exert their action by binding to tyrosine kinase receptor and p75 neurotrophic receptor (NTR). BDNF exerts a stronger effect when binding to tyrosine kinase B (TrkB) receptor, whereas NGF when binding to tyrosine kinase A (TrkA) receptor (Salama-cohen *et al.*, 2005; Yamada and Nabeshima, 2003). In this study, hippocampal BDNF and NGF were assessed at the protein level. To observe the epigenetic effects of aerobic exercise and environmental enrichment, we are currently conducting a study to assess the mRNA expression of hippocampal BDNF and NGF.

BDNF is a neurotrophin (NT) involved in synaptic plasticity and survival of neurons. It accomplishes these functions by promoting proliferation, survival and differentiation of neurons in the peripheral and central nervous systems (Yamada and Nabeshima, 2003). Even though BDNF is more concentrated in the brain, it is also present in the blood and is produced in the periphery from different sources which includes the liver, skeletal muscles, and blood (haemoglobin and plasma) (Lommatzsch *et al.*, 2005). Studies have shown that BDNF can cross the blood-brain barrier and there is also positive correlation between peripheral and brain BDNF protein levels (Karege *et al.*, 2002). Several data provide the linkage between BDNF and memory. First, BDNF plays a key role in the late LTP phase, the best-known phenomenon for cellular plasticity in the brain. It has been shown that BDNF is required for late LTP phase (L-LTP) in hippocampal CA1 region (Bekinschtein *et al.*, 2011). Secondly, BDNF mRNA and its protein expression is induced after learning (Bekinschtein *et al.*, 2011). Third, the effect of BDNF on structural plasticity is similar to the effects correlated with learning (Bekinschtein *et al.*, 2011). The results of this study showing the highest BDNF concentration in EEA group is supported by previous studies on the effect of aerobic exercise and continuous EE exposure on memory function (Abdullah, 2017; Dita, 2017).

NGF is a family of small secreted protein that play essential roles in maintaining the physiology of neurons through regulating their survival, growth, and differentiation, as well as synaptic formation, plasticity, and other associated action of neurons throughout the development processes (Itakura *et al.*, 2013). A recent finding provided the first direct evidence that the availability of NGF can affect hippocampal physiology and behaviour in normal adult brain by modulating the brain's cholinergic system. Selective augmentation of septohippocampal function with NGF significantly facilitates hippocampal plasticity, where blockade of NGF weakens hippocampal plasticity. Additionally, blockade of NGF significantly damages memory retention (Conner *et al.*, 2009). The results of this study showed that exposure of adult rats to combination of aerobic exercise and continuous environmental enrichment for eight weeks induced the highest levels of hippocampal NGF. Previous study reported higher hippocampal NGF levels in animals located in environmental enrichment model (Ickes *et al.*, 2000); this is in line with our study.

5. Conclusions

In conclusion, both aerobic exercise and environmental enrichment by themselves increases hippocampal BDNF and NGF and memory function, but combination of aerobic exercise and continuous EE exposure produces the highest levels of hippocampal BDNF and NGF and improves memory function the best in adult rats.

Supplementary material

Supplementary material can be found online at https://doi.org/10.3920/CEP190036.

Materials and methods S1. Tissue sampling method.

Conflict of interest

The authors declare no conflict of interest.

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