RESEARCH ARTICLE

Environmental Enrichment and Aerobic Exercise Enhances Spatial Memory and Synaptophysin Expression in Rats

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Abstract

BACKGROUND: Exposure to environmental enrichment has a positive effect on brain function, including improved cognition. Environmental enrichment has many aspects, including social interactions, object stimulations, and physical activities. Exercise and environmental enrichment can be considered to improve cognitive function with different underlying mechanisms. This study aims to compare the effects of environmental enrichment and aerobic exercise at both synaptic and whole-organism levels using synapyophysin as a measure of synaptic physiology and spatial memory as a measure of cognitive function.

METHODS: A six-week *in vivo* experimental study on 15, 6-month old male Wistar rats randomly divided into three groups (n=5): aerobic group (A), enriched environment group (EE), and enriched with an aerobic or combined group (EEA). All rats were tested four times in the Water-E maze (WEM) task at weeks 0, 2, 4, and 6 of the study. We used immunohistochemistry to determine the synaptophysin expression in hippocampal CA1 region.

RESULTS: Based on synaptophysin immunostaining, there were higher optical density scores for synaptophysin in hippocampal CA1 region following EEA, but there were no statistically significant differences between groups (ANOVA test, $p>0.05$). The spatial memory test showed there were significantly reduced travel time and total errors from the 2nd and 4th weeks in the EEA group, respectively ($p<0.05$).

CONCLUSION: The combination of enriched environment and aerobic exercise seems to rapidly improve spatial memory and enhances the presynaptic protein, synaptophysin in hippocampal CA1 region.

KEYWORDS: aerobic exercise, environmental enrichment, spatial memory, synaptophysin, Water-E maze

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Introduction

The memory decline is associated with decreasing neuroplasticity along with the aging process. Learning and memory, which directly relate to experience-dependent plasticity and increased neurogenesis, can be improved by physical exercise induction.(1) In addition to physical exercise, there are factors that affect synaptic plasticity and neurogenesis including social interaction and behavior stimulation.(2) Therefore, a model that combines physical activity with other forms of stimulation like environmental enrichment has been developed. In the environmental enrichment model, animals are exposed to object stimulation and social interaction which are greater and better than the exposure in standard conditions.(2) Environmental enrichment provide an arena for animals to exercise, play and explore, including

running-wheels that provide opportunities to increase physical activity.(2,3)

Neurogenic stimuli can come from exercise which enhances cell proliferation, and environmental enrichment which increases new cell survival.(3) Although through different mechanisms, exercise and enrichment cannot be completely separated. Enrichment without running wheels cannot increase neurogenesis in the hippocampus of mice. (2) The inclusion of running wheel in environmental enrichment in this experiment aims to increase physical activity of rats. However, running wheel is a voluntary exercise for the rats with dependent factors of exercise such intensity and frequency have been unknown. Meanwhile, studies have shown exercise to be the most effective method of improving cognitive function and brain health. Animal studies have shown that moderate-intensity exercise has a positive effect on memory function.(4-7)

Both aerobic exercise and environmental enrichment have been reported to increase the amount of presynaptic proteins involved in the hippocampal synapse formation in rats.(8-10) One of the presynaptic proteins is synaptophysin. Synaptophysin, which serves as a common marker for synapse activity, is a major integral membrane protein of presynaptic vesicles required for vesicle formation and exocytosis.(11) In cultures of hippocampal neurons, synaptophysin is involved as a regulator in the activity-dependent synapse formation.(12) Furthermore, loss of hippocampal synaptophysin has been previously correlated with cognitive decline such as Alzheimer's Disease, emphasizing a possible connection between synaptophysin and cognitive functioning.(13)

Therefore, the current study aims to determine whether the combination of aerobic exercise and environmental enrichment can provide a greater effect on spatial memory and increase synaptophysin expression compared to aerobic or environmental enrichment-only treatment.

Methods

Subjects

Fifteen male Wistar rats from Balai Penelitian dan Pengembangan Kesehatan RI (Center of Health Research and Development of Indonesia, Jakarta) were used. They were moved from an animal facility into the laboratory for 2 weeks and then handled for 2 weeks prior to testing to habituate them to handling by the experimenter. Subjects were housed in groups. All rats had ad libitum access to food and tap water, and were maintained at constant room temperature (20-21°C) with artificial light-dark cycle of 12 hours (08:00-20.00 hour dark/20:00-08.00 hour light). The experimental protocols applied in this research were previously reviewed and approved by the ethics committee of Universitas Indonesia (849/UN2.F1/ ETHICS/2016).

The rats were 6 months old at the start of the experiment and at the beginning of spatial testing. The rats were randomly assigned to three groups: the aerobic group (A; n=5), enriched group (EE; n=5), and enriched with an aerobic or combined group (EEA; n=5). Aerobic rats were housed in standard cages and treated with aerobic exercise. Enriched rats were housed together in a group of ten in an environmental enrichment cage. The rest of the day, enriched rats were housed in groups of five in standard cages without stimulating objects. Combined rats performed the same EE protocol as the enriched groups and were then treated with aerobic exercise. All groups were handled, habituated, and trained in the Water-E maze (WEM) task to measure spatial memory.

Aerobic Exercise

Exercise is an activity done to maintain one or more components of physical fitness by practicing repetitive body movements. Exercise given to the aerobic and EEA groups was of moderate intensity physical exercise using animal treadmill. These rats exercised for 30 days, 30 minutes a day, 5 times a week at 20 m/minute in the middle of the active cycle.

Environmental Enrichment

Marlau cages were used for environmental enrichment conditioning in this experiment. EE and EEA groups were housed in Marlau cages for 30 days, three hours/day, five days a week (10.00-13.00) Daily limited exposure to environmental enrichment is known to positively affect stress response and cognition.(14) The rats were then housed in groups of five in standard cages without stimulating objects for the rest of the day. The Marlau cage (800 x 600 x 510 mm) is a standardized EE cage, which exposes the rats to challenges requiring the use of cognitive skills in order to survive and thrive. This was made possible due to the design of the Marlau cage that is divided into two floors. The ground floor was divided into two parts, the first part (G1; 296 x 600 mm) had food pellets, and the second part (G2; 496 x 600 mm) had 3 water bottles, a rectangular house with 4 lateral windows; and 3 running wheels. G2 was connected to the upper floor via a ladder. The upper floor, which contained a maze, was connected to the G1 via a slide tunnel. When the rats were in G1, they could move to G2 using two doors that

open to only one direction. To get food, the rats must climb the ladder from G2 to the upper floor, find their way in the maze and go down to the G1 via the slide tunnel. To access water, the rats could use one-way doors placed on a plastic wall between G1 and G2. The rats, repeated this procedure each time they needed to eat.(14)

To ensure novelty, the configuration of the maze was changed 3 times a week (Monday, Wednesday, and Friday) using six maze (series A-F), each consist of two different configurations.(14) The cage was cleaned twice a week. Video records were taken throughout the environmental enrichment condition to ensure that all rats made the same use of environmental enrichment elements. At the end of behavioral testing, the rats had been enriched for 30 days.

Water E-Maze (WEM) Task

An E-shaped glass pool was filled with water at a temperature of 24±2°C to a depth of 26.5 cm. The glass pool consisted of one main trench (u) and three other trenches branching perpendicularly to the main trench, two edge trenches (ti) and one middle trench (ta). The width of each trench was 25 cm and the height 60 cm. The starting point of WEM placed in the middle of the trench (ta) and the target point was at one of the trench edges (ti) where the ladder was located.(15)

WEM task was done every two weeks at week 0, 2, 4, and 6. Twice a week, the rats received a habituation session during which they were given one trial in the WEM. WEM task, which included three trials without rest, was performed every two weeks at the end of week 2, 4, and 6. Each rat was allowed 120 seconds to reach the ladder. If the rat failed, they would be guided to the ladder and allowed to remain there for 10 seconds. At the end of the testing, the rats were dried and returned to their home cage. Multiple measures of water maze performance were recorded. Travel time (s) and total errors (times/test) were monitored and assessed during three trials. Shorter travel time and fewest number of total errors indicated better performance.

Protein Immunostaining

The rats were decapitated before having their brains removed immediately. The brain tissues were fixed with 4% paraformaldehyde until they became paraffin-embedded. Immunohistochemistry (IHC) assay was then performed using the paraffin sections of the hippocampus (4 µm) to identify synaptophysin expression.

The brain sections were incubated with rabbit anti-synaptophysin monoclonal antibody (Abcam, Cambridge, UK) (1:1200) including 0.3% Triton X-100 for 1 hour at room temperature. The brain sections were being rinsed in 0.01 M PBS for 5 minutes. The rinsed brain sections were incubated firstly with biotinylated secondary antibody (Biocare Medical, California, USA) for 15 minutes, and secondly with avidin-biotin peroxidase (Biocare Medical, California, USA) complex for 15 minutes at room temperature. Enzyme-substrate kits Star Trek Universal HRP detection system (Biocare Medical, California, USA) was then used to visualize the immunoreactivity. The rat cerebral cortex is used as positive control. Negative control sections were treated using the same method, but without the anti-synaptophysin antibody.

The sections were then counterstained with hematoxylin. For synaptophysin immunostaining, various sections of the hippocampal CA1 region were measured under a light microscope (DM500, Leica, Wetzlar, Germany) equipped with a built-in camera (ICC50 HD, Leica) at a magnification of 400x. Synaptophysin expression was analyzed using a computer-associated image Analyzer software Image J (NIH, Maryland, USA). It is open-source plugin for IHC analysis using color deconvolution and computerized pixel profiling to enable assignment of an automated scoring of each image. The final score shown a semiquantitative grade (negative, low positive, positive, and high positive). Using algebraic formula, IHC OD score was calculated.(16)

Data Analysis

Behavioral data and Immunostaining data were analyzed with SPSS 21.0 and were expressed as mean±standard deviation. The results were considered statistically significant if $p<0.05$ and were represented graphically with Prism (GraphPad, San Diego, USA). For the water maze, behavioral test measures (travel time and total errors) were averaged within a group for each test and analyzed using the Friedman test followed by Wilcoxon Post-hoc test with the group as an independent variable. Synaptophysin data were analyzed using one-way ANOVA for each hippocampal CA1 region.

Results

Subjects

All rats appeared in good health. For behavioral analyses, we used 15 subjects: aerobic group (n=5), enriched group (n=5), and combined group (n=5). For the histological analyses, we used 11 rats from the initial sample of 15 subjects because the other 4 were neurochemical outliers

as defined by technical errors during staining such as poor staining, and thus, the sample sizes for the synaptophysin expression were: aerobic group (n=4), enriched group (n=3), and combined group (n=4).

Spatial Memory

Spatial memory test in this study using WEM tasks is presented in Figure 1A and 1B. Figure 1A and 1B illustrates a decrease in travelled time and total errors of each group. Wilcoxon post-hoc test exhibited a statistically significant decrease in travelled time per week 2 (Z=-2.023, p=0.043) and total errors per week 4 (Z=-2.023, p=0.043) in the combined group. Meanwhile, Wilcoxon post-hoc test exhibited a statistically significant decrease in travelled time per week 4 (Z=-2.023, p=0.043) and total errors per week 6 (Z=-2.060, p=0.039) in the aerobic group. No significant difference was observed in the enriched group.

Synaptophysin Immunostaining

Representative photographs of synaptophysin immunostaining in the hippocampal CA1 region are shown in Figure 2. Comparison of aerobic, EE, and EEA groups suggested there were higher optical density scores of synaptophysin in hippocampal CA1 region following EEA, but this was not significant.

Discussion

This study shows that improved spatial memory occurs faster in the groups that included animal treadmill (A and EEA) than EE group which suggests that exercise become an important factor involved in synaptic plasticity, and promote memory formation.(17) Learning and memory improved by exercise are directly related to experience dependent-plasticity, changes of gene expression, and increased neurogenesis.(1) This agrees with a previous study mentioning aerobic exercise as an important stimulus for environmental enrichment, which showed increased neurogenesis and furthermore, changes in morphology and function of the hippocampus.(17) In line with these changes, aerobic exercise and exposure of environmental enrichment led to improved performance in various hippocampal dependent-memory tests such as spatial memory.(2,18,19)

This study aims to assess the effects of environmental enrichment and aerobic exercise on spatial memory in a Water E-maze and the expression of presynaptic marker, synaptophysin. All the rats in this study were treated with aerobic exercise and exposed to 30 days of environmental enrichment prior to testing in the Water E-maze. Behavioral testing showed that rats which experienced combined

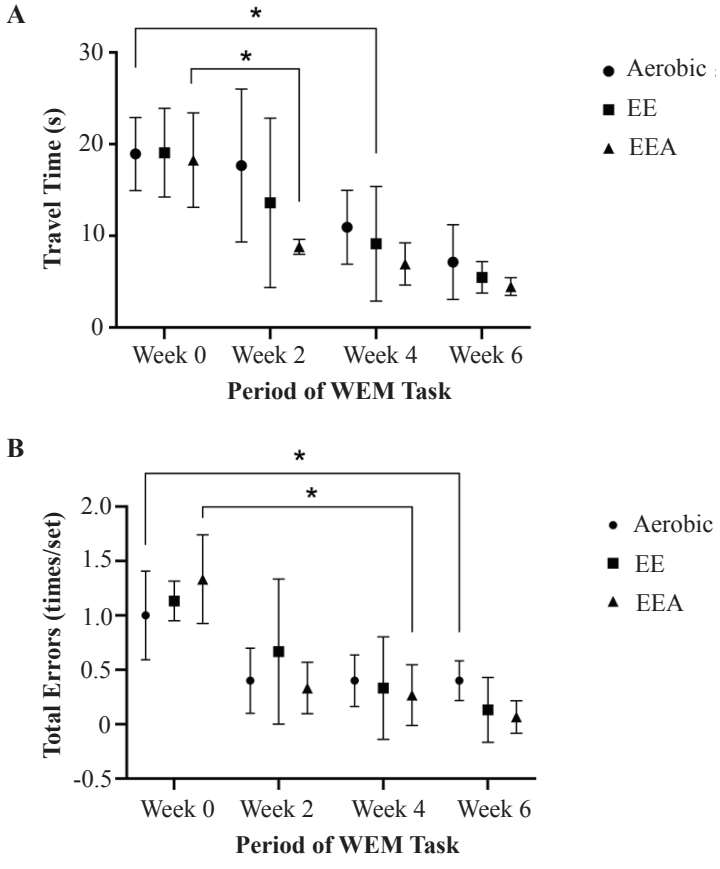

Figure 1 . Spatial memory test using WEM task. Travel time (A) and total errors (B) were recorded during water-E maze task (trials 3 of each test). Each point represents the mean±standard deviation (SD) of each group. *p<0.05.

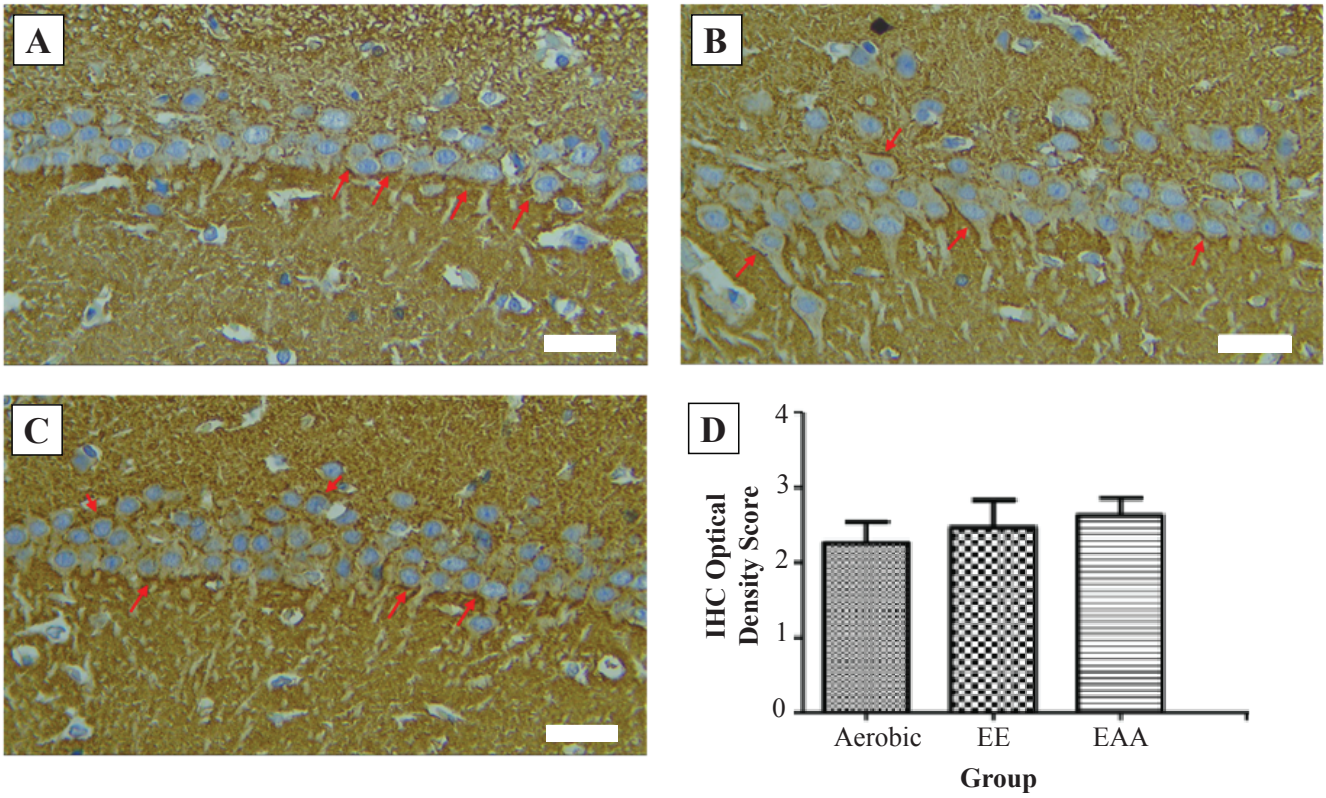

Figure 2. Synaptophysin expression (red arrow) in the hippocampal CA1 region. A: Aerobic group; B: EE group; C: EEA group; D: Optical density quantification of each groups. Scale bar 10 µm.

environmental enrichment and aerobic exercise performed better during testing on Water E-maze, as measured by travel time and total errors. This finding suggests that the combination of enrichment and aerobic exercise seem to have improved spatial memory related to memory consolidation. Memory consolidation is related to synaptic plasticity phenomena. The measure of the travel time and total errors in the first test before an experiment, also in the first trial of each test, allow us to test the memory consolidation ability of the rats. All rats showed an improvement in their performance as the day progressed (Figure 1A and 1B).

Comparing all groups on spatial memory, there were no significant differences in travel time and total errors. It showed that physical activity along with other stimuli have an equivalent effect as regular exercise. Despite the different underlying mechanisms, exercise and EE have been regarded as equivalent neurogenic stimuli. Exercise can enhance cell proliferation, while enrichment can enhance new cell survival.(3) Running, using the Morris water maze, is shown to improve learning and memory.(20) Another study using the Morris water maze indicated that aerobic exercise can reduce travel time to reach the platform.(5) This suggests that exercise may improve learning ability. The complexity of physical activity such as enrichment can also improve learning and memory.(18) Environmental enrichment was able to improve memory consolidation in young animals as seen in the short distance to reach the target.(2) Our results are consistent with previous studies showing a combination of aerobic and enrichment resulted in better habituation within the open field test.(17)

The hippocampus has been intensively studied in learning and memory (10), because it has a dynamic brain structure that is critical to the process of memory consolidation (3). Environmental enrichment is associated with the reorganization of a number of neurons in the hippocampus. Our results show that synaptophysin immunostaining was increased by a combination of environmental enrichment and aerobic exercise in hippocampal CA1 region. Because synaptophysin is a constituent of neurotransmitter-containing presynaptic vesicle membranes (21), an increase in synaptophysin may reflect an increase in synapse number, or capacity of existing synapses for neurotransmission. Although only a correlation, it suggests, that the increase in neurotransmission may lead to improved spatial memory. Our findings agree

that treadmill exercise elicits an enhancing effect on the expression of these hippocampal proteins of normal rats. (22) Increased synaptophysin expression in normal rats after a short period (7 days) of treadmill exercise has also been reported.(9) Several studies have shown an increase in hippocampal synaptophysin levels in rats through voluntary exercise.(8,23,24) In addition, experimental evidence also suggests a positive correlation between synaptophysin and brain-derived neurotropic factor (BDNF).(25,26) In the hippocampus of BDNF-knockout mice, synaptophysin levels were markedly decreased.(25) The increase of exercise-induced synaptophysin levels could be stopped by blocking BDNF.(26)

In this work we have demonstrated that aerobic exercise is an important stimulus in environmental enrichment and is associated with alterations in hippocampal synaptophysin expression of hippocampal-associated cognitive ability. It is thus conceivable that forms of environmental enrichment combined with aerobic exercise could be effective in improving spatial memory. This research can be implemented in early childhood by modifying the learning method in school along with other forms of physical activity and physical exercise.

Using *in vivo* study, we have shown that environmental enrichment combined with aerobic exercise seems to have shorter travel time and total errors to reach the ladder. This suggests that environmental enrichment combined with aerobic exercise seem to have rapid effects on spatial memory. The synaptophysin results suggested an increased expression in hippocampal CA1 region after treatment with environmental enrichment combined with aerobic exercise. This suggests that environmental enrichment and aerobic exercise can modulate levels of presynaptic proteins in the brain.

Conclusion

Environmental enrichment combined with aerobic exercise is better to improve spatial memory and induce synaptophysin expression than aerobic exercise or environmental enrichment alone.

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