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Voluntary Diving Exercise Improves Hippocampus-dependent Learning in Rats

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ABSTRACT

Several physiological alterations that arise in response to hypoxemia conditions when diving are intended to protect oxygen-sensitive organs from the hypoxic condition. Hippocampus, known for its central function in memory formation, is sensitive to hypoxic conditions. This research aimed to study the effect of voluntary diving exercise (VDE) on the hippocampus-dependent learning performance of rats. Rats were divided into control and diving groups, with swim and voluntary diving exercises every day for 60 days, respectively. Observation of memory consolidation was carried out using Morris Water Maze (MWM) and Novel Object Recognition (NOR) tests. In the MWM test, the escape latencies in the diving group were shorter than those in the control group. Consistent results were obtained in the MWM probe trial which the time spent in the target quadrant was significantly longer in the diving group. Moreover, the diving group spent more time exploring the novel object in the NOR test. Based on the results, we can conclude that 60 days VDE significantly improves hippocampus-dependent learning capacity in trained rats.

Keywords: learning; memory; novel object; voluntary diving; water maze

INTRODUCTION

Feral rats (*Rattus norvegicus*) can naturally dive to search for underwater food in their habitat (McCulloch, 2012). They exhibit a physiological strategy to overcome breathing cessation during diving called diving response (Panneton, 2013). Diving causes low levels of oxygen in the blood or hypoxemia condition (Ridgway & McFarland, 2006). On the other hand, the brain consumes 20% of the body's total oxygen consumption under normal condition (Spiotta *et al*., 2010; Fan *et al.*, 2012), even though the brain only makes up 2% of the total body weight (Michiels, 2004). Furthermore, brain sensitivity on oxygen partial pressure changes is quite high, this sensitivity is mediated by astrocytes (Angelova *et al.*, 2015; Marina *et al*., 2018; Takata *et al*., 2018).

Cell-level physiological response mechanism to hypoxic conditions is mediated by hypoxia-inducible factor (HIF-1) that plays a role in the regulation of the transcription level of other genes involved in the response to hypoxia: erythropoiesis-coding genes (Keswani *et al.*, 2011; Agani *et al*., 2013), Fe-metabolism (Vigani, 2012; Guo *et al*., 2015), angiogenesis (Ahluwalia & Tarnawski, 2012; Ahn *et al*., 2014), aerobic glycolysis (Stubbs & Griffiths, 2010; Semba *et al*., 2016), and growth factor (Wenger, 2002). Specific in the brain, hypoxic conditions lead to neuroglobin upregulation that possibly increases oxygen supply to the mitochondrial nerve cells (Burmester & Hankeln, 2009).

Part of the brain that susceptible to hypoxic conditions is the hippocampus which plays an important role in memory formation (Ridgway & McFarland, 2006; Macri *et al*., 2010). Memory formation consists of three distinct mechanisms: encoding, consolidation, and retrieval of the information (Straube, 2012). Scientists have divided memory into several distinct memory systems based on how our brain process the information: semantic, episodic, working, and procedural memories (Matthews, 2015). In recent research, we assessed spatial and recognition memory as part of the episodic memory category.

Several investigations have been conducted to elucidate brain protection against hypoxic conditions using forced submersion animals (Panneton *et al*., 2010). It has been questioned that the hypoxic conditions in forced diving can affect the diving responses.

However, the effect of prolonged voluntary diving exercise on memory function is still unknown. Therefore, this research aimed to investigate the memory consolidation performance in rats after 60 days of voluntary diving exercise (VDE).

MATERIALS AND METHODS

Animals, housing, and ethical statement. Female rats (*Rattus norvegicus* Berkenhout, 1769) Wistar strain from Universitas Gadjah Mada Integrated Research and Testing Laboratory were acclimated at the animal facility in the Faculty of Biology, Universitas Gadjah Mada. Rats were housed in standard cages (40×30×20 cm) with standard pellet food and tap water ad libitum. The cages contained wood-chip bedding and were maintained in constant temperature (26-27°C) and humidity (76-88%) on a 12 hour light/dark cycle. The animal experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) of Universitas Gadjah Mada (Ref. 00059/04/LPPT/V/2018).

Animal Treatment. Twenty female rats were divided into control (CNT) and treatment (DIV) group. The voluntary diving exercise was carried out by training rats to dive periodically for 60 days (five days in a week). Meanwhile, CNT rats were trained to swim in similar distances with DIV rats. The diving chamber was built from glass material with 100×30×20 cm^3 dimension, separated into twin connected tracts (total 200 cm length). The chamber was filled with tap water and maintained at 30-32°C. In the diving exercise, the water surface was covered with plexiglass to avoid the rats inhaling surface air. Meanwhile, in the swimming exercise (CNT), the cover was opened. Rats were trained to dive constantly for 200 cm length. As the introduction to start module, rats were lowered slowly using a manual elevator. Rats were then left to exit and swim to the finish module. After several days, the water level was raised to cover the exit way of the start module. Diving training completed when rats can escape from the start module then dive into the finish module (Figure 1).

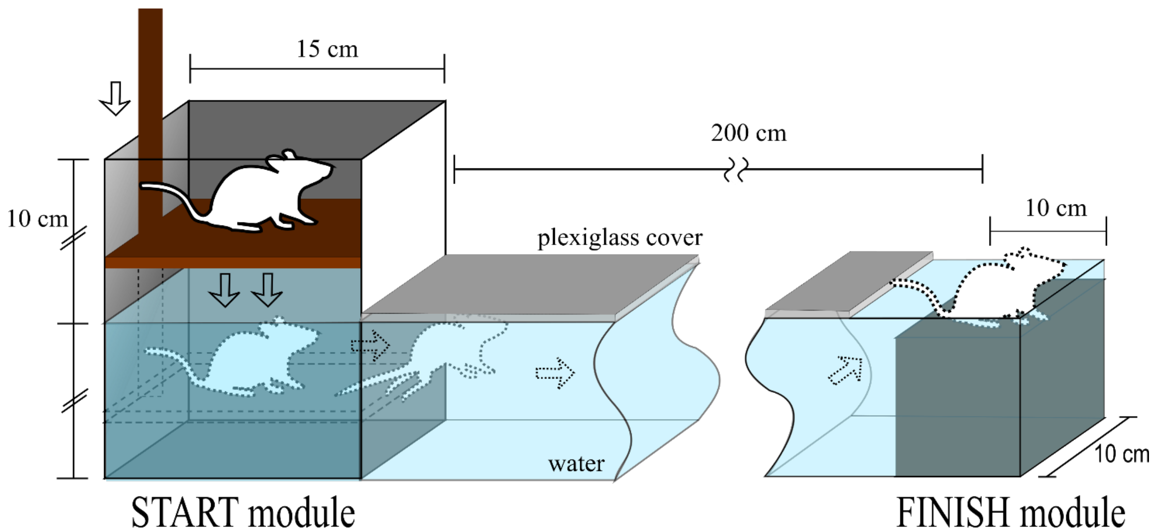

Figure 1. Rats VDE apparatus design. Start module design consisted of a manual elevator and a submerged exit way. The diving/swimming distance was 200 cm length, from the start module to the finish module.

MWM tests. Morris water maze (MWM) tests were conducted using a black-colored circular tank (diameter: 150 cm; height: 50 cm) filled with tap water (30-32°C). The start or finish platform was created from clear glass (10 x 10 x 15 cm). Rats swimming trajectories were recorded using B-PRO5α fixed focus F2.8 f= 3 mm 170o wide-angle lens (Brica, China) then computed using Idtracker 2.1 (Cajal Institute, Madrid, Spain). We measured duration of rats exploring novel and familiar objects using the Preference Index (PI) i.e. a percentage of the time spent by rats exploring novel objects divided by total exploration time. The MWM tests were carried out for five consecutive days in sequence: training trial, probe trial, and visual cue trial (Vorhees & Williams 2006). On the first day training, the rat was released in the tank at four random points with a visible platform (above the water). On day two until four, training trials were conducted with the hidden (submerged) platform. Escape latency for each trial was recorded from release time until rats reach the finish platform. On day 5, a probe trial task was performed by allowing to swim in the tank for 60 s without the platform. The duration spent in the target/platform quadrant was recorded. Subsequently, a visual cue test was performed, the platform was returned into the visible condition and the escape latency was recorded.

NOR tests. Novel object recognition (NOR) tasks were carried out for four consecutive days: familiarization, habituation, and test day (Antunes and Biala, 2012). In familiarization, a rat was released in the test box (40 x 40 x 40 cm, black colored) for five minutes. Habituation was conducted by placing two identical objects in the box for five minutes duration on two consecutive days. Afterward, the test day was performed by replacing one object with another novel object for 60 s. The time spent by the rat to explore the novel object divided with total exploration time was determined as a preference index (PI).

Statistical Analysis. All data were analyzed using SPSS 22.0 (IBM corporation, USA) for Independent T-test, ANOVA, Mann-Whitney U test, and Tuckey test. Tests were considered significant at $p < 0.05$.

RESULT AND DISCUSSION

Spatial memory is correlated to brain function in preserve information about the location of any physical stimuli regarding body position (egocentric) and external space (allocentric) (Paul, et al., 2009). In the MWM tests, rats create a spatial memory using external visual information around the chamber. Memory was assessed by the duration before the animal find the platform (escape latency) and by the percentage of the time spent in the target quadrant. During day-5 MWM test, the escape latencies of DIV rats (5.27 ± 0.43 s) were shorter than those in CNT rats (6.77 ± 1.85 s). The results of the Mann-Whitney U test showed there was no significant difference in escape latency between groups at day-5 MWM ($p > 0.05$) (Figure 2a).

Subsequently, a probe trial was conducted on day-5 training for one minute total duration to assess spatial memory. Swimming trajectories of rats at the MWM probe trial showed normal movement path of CNT and DIV rats along the target quadrant (Figure 3a). However, the time spent in the target quadrant of DIV rats (46.32 ± 1.80 %) were significantly longer than those in CNT rats (34.85 ± 3.34 %) ($p < 0.05$) according to Independent T-test (Figure 3b).

To assess that the results of MWM tests on memory performance were not affected by sensorimotor function, we performed the MWM visual cue test. The test was conducted after the MWM probe trial on day-5 of training. Statistical analysis using the Mann-Whitney U test showed no significant result of escape latencies in CNT and DIV rats during MWM visual cue test (Figure 2b).

We used MWM tests to assess hippocampus-dependent learning without being affected by external motivation such as food. The escape latencies in the DIV rats were shorter than those in the CNT rats, indicating that the learning capacity was increased after subchronic VDE. Moreover, consistent results were obtained in MWM probe trial, in which the time spent in the target quadrant was significantly longer in the diving group. The sensorimotor factor can be ignored because no significant result has been found in the MWM visual cue tests.

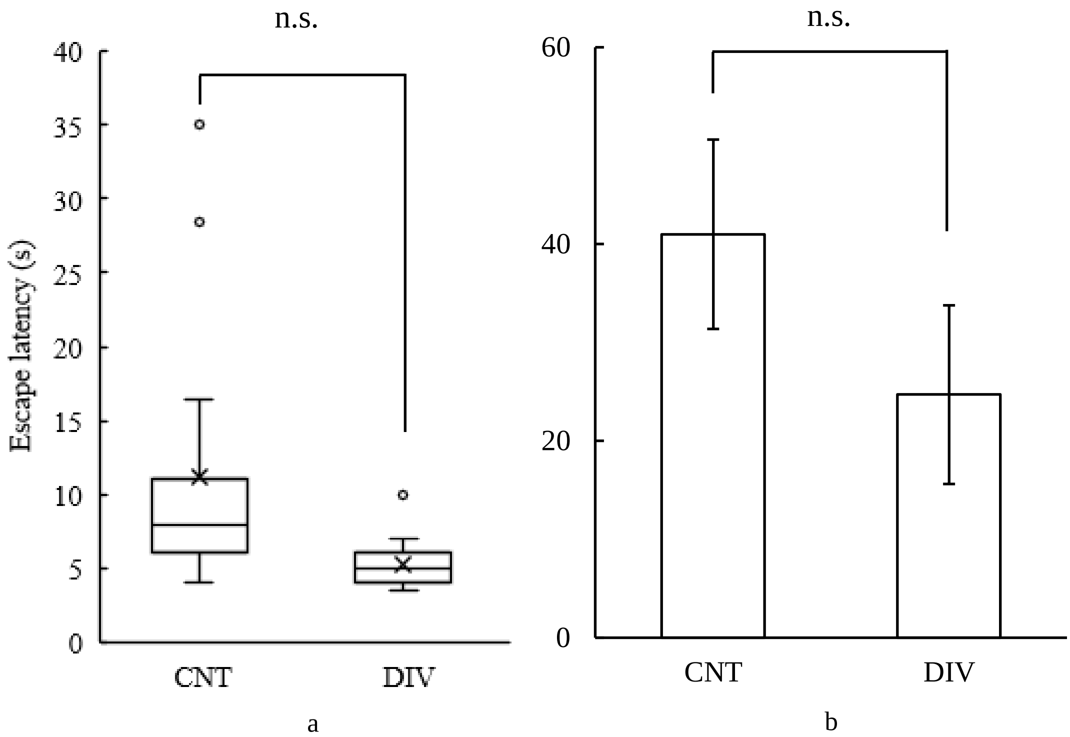

Figure 2. Performance of CNT and DIV rats during day-5 MWM test and MWM visual cue test: a. Escape latency of DIV rats was shorter than CNT rats at day-5 MWM test using invisible platform but statistically no significant (n.s., $p > 0.05$, Mann-Whitney U test). Data are presented as mean (×) and median with upper and lower quartiles, min and max values and outliers (o). n=5 for all groups; b. MWM visual cue test showed no significant result of CNT and DIV rats (n.s., $p > 0.05$, Mann-Whitney U test). Data are presented as mean ± S.E.M. n=5 for all groups. CNT: control group; DIV: diving group.

In addition to the MWM, the rats were subjected to NOR tasks, which also evaluate hippocampus-dependent learning, especially recognition memory. Recognition memory is the capability to consider whether a recent object has been presented before, this system consists of two parts: recollection and familiarity (Squire et al., 2007). We observed the duration of rats exploring novel and familiar objects. PI of DIV rats (85.11±14.88%) were significantly higher than those in CNT rats (55.46±10.42 %), based on Independent T-test ($p < 0.05$) (Table 1). It means that the DIV rats spent more time exploring the novel object. Furthermore, rats of all groups have sufficient motivation to learn, since there is no significant result in the total exploration time ($p > 0.05$).

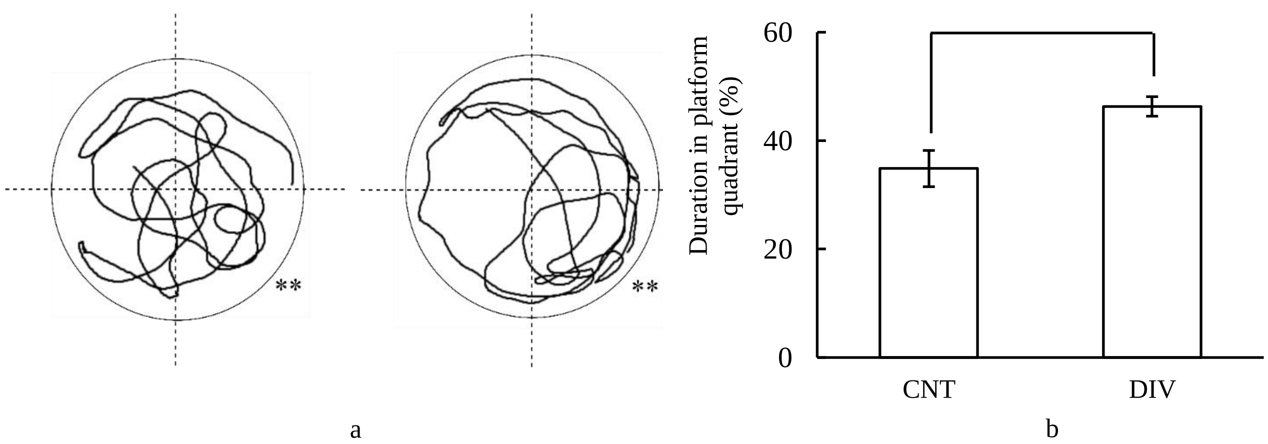

Figure 3. Swim trajectories and time spent in the target quadrant during MWM probe trial: a. Representative example of individual swimming trajectories of CNT and DIV rats during MWM probe trial using a circular chamber (150 cm in diameter). Asterisk (**) indicates the quadrant of escape platform position that have removed in this probe trial; b. Time spent in the target quadrant of DIV rats was significantly shorter than those in CNT rats at MWM Probe trial ($p < 0.05$, Independent T-test). Data are presented as mean ± S.E.M. n=6 for all groups. CNT: control group; DIV: diving group.

Rat physiological responses to diving are different than those in swimming. In the diving state, they exhibit a decrease in arterial pressure and heart rate (Hult *et al*., 2019). Therefore, a diving rat is susceptible to hypoxemia condition i.e. low oxygen level in their blood. Hypoxemia condition is not always related to brain hypoxia (Ridgway & McFarland, 2006). Therefore, brain cell necrosis related to hypoxemia may not occur unless in prolonged dive. Hooded Seals (a diving mammal) show a lower aerobic capacity of the brain when they undergo long dives, which may be assumed as an energy-saving strategy (Fabrizius *et al*., 2016).

Table 1. Preference index and exploration time of CNT and DIV rats during NOR test

Groups	**NOR parameters**	
	Preference Index (%)	**Exploration time (s)**
CNT	55.46 ± 10.42	12.93 ± 5.54
DIV	85.11 ± 14.88 *	13.27 ± 3.46 n.s.

CNT: control group; DIV: diving group; *: significant to CNT ($p < 0.05$ Independent T test); n.s.: not significant to CNT ($p < 0.05$ Independent T test)

VDE mimics an intermittent hypoxia condition in rats' brains. As the diving duration in the current research was short, neurons in the brain may only undergo transient hypoxia. Our results showed that this condition improves memory performance in rats and is consistent with previous research about an improvement of memory in rat's hippocampus (Bouslama *et al*., 2015).

Repetitive acute intermittent hypoxia also increases neurotrophic factors in motor neuron (Satriotomo *et al*., 2016). Furthermore, neural plasticity is modulated by neurotrophic factors such as brain-derived neurotrophic factor (BDNF)(Castrén & Antila, 2017). The brain conducts plasticity through various stimuli regarding learning and memory which modifies the brain structures, functions, and connections (Mateos-Aparicio & Rodríguez-Moreno, 2019). However, further investigations need to be conducted to elucidate molecular and neurophysiological mechanisms underlying the increase of memory consolidation performance due to repeated hypoxia exercise.

CONCLUSION

Based on the results, it can be concluded that the subchronic voluntary diving exercise indicates improvements in hippocampus-dependent learning in rats.

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