THE CONSEQUENCES OF CHRONIC UNPREDICTABLE STRESS EXPOSURE TO SPATIAL MEMORY OF ADOLESCENT MICE (*Mus musculus albinus*)

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BOGOR
2018
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ABSTRACT

NIA CHRISTIN. The Consequences of Chronic Unpredictable Stress Exposure to Spatial Memory of Adolescent Mice (Mus musculus albinus). Supervised by BERRY JULIANDI, ARIEF BOEDIONO, and NORIAKI SASAI.

Adolescence is a sensitive period when neurogenesis rate is increases. Chronic stress that occurs during adolescence can cause a long-term effects on adult behavior and morphology of hippocampus. Light-dark transition test showed that CUS successfully produced depressed adolescent mice. Y-alternation test revealed that short-term effect of CUS improved spatial memory in mice, resembled the capability of non-stressed adult while cell counting performed with HE staining did not showed a significant effect on proliferation, cell maturing, and cell survival in DG. Complexity of dendrites in DG and pyramidal neurons of CA1 performed using Golgi-Cox staining. Apical dendrites of DG did not showed a significant effect of CUS on dendritic complexity of DG. In contrast, both of apical and basal dendrites of CA1 showed a significant short-term effect of CUS, seen from the number of intersections. Maximum dendritic span and total dendritic length did not show a significant differences. It is remarkable that both short and long-term effect of CUS is changes the complexity of CA1 pyramidal neuron and transciently increases spatial memory on adolescent mice.

Keywords: chronic unpredictable stress, dendritic complexity, Golgi-Cox, neurogenesis, spatial memory

ABSTRAK

NIA CHRISTIN. Dampak Perlakuan Chronic Unpredictable Stress terhadap Memori Spasial Mencit (Mus musculus albinus) Remaja. Dibimbing oleh BERRY JULIANDI, ARIEF BOEDIONO, dan NORIAKI SASAI.


Kata kunci: chronic unpredictable stress, Golgi-Cox, kompleksitas dendrit, memori spasial, neurogenesis
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THE CONSEQUENCES OF CHRONIC UNPREDICTABLE STRESS EXPOSURE TO SPATIAL MEMORY OF ADOLESCENT MICE (*Mus musculus albinus*)

NIA CHRISTIN

An Undergraduate Thesis
Intended to Acquire Bachelor Degree
In Department of Biology

DEPARTMENT OF BIOLOGY
FACULTY OF MATHEMATICS AND NATURAL SCIENCES
BOGOR AGRICULTURAL UNIVERSITY
BOGOR
2018
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FOREWORDS

First of all, I would like to give thanks to God for His goodness that this undergraduate thesis can successfully finished. The selected theme in the research that conducted since September 2017 is The Consequences of Chronic Unpredictable Stress Exposure to Spatial Memory of Adolescent Mice (Mus musculus albinus).

Deepest acknowledgements to Dr Berry Juliandi M.Si, Prof drh Arief Boediono, and Prof Noriaki Sasai as my supervisor for their advice and supervision. Another acknowledgements are given to Dr Ir Gayuh Rahayu as the examiner of my undergraduate thesis. Special acknowledgements are also extended to my beloved parents and sisters for all their prayer and support. I also express my thankfullness to all BJL research students, Zoo Corner Family for all of the advice, Mr. Adi Surahman and Mrs. Tini Wahyuni as laborant for their help during this research, and all of my friends in Department of Biology for the support and togetherness.

Finally, I hope this research will be helpfull for all readers.

Bogor, September 2018

Nia Christin
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INTRODUCTION

Background

Adolescence is a sensitive period of life that marked by puberty (Fuhrmann et al. 2015). During this time, brain undergo a significant remodeling through increases of neurogenesis rate and synapse overproduction, followed by rapid pruning of existing sinapses and neurons. This process is called brain maturation that especially occured in cortical regions and limbic system (Andersen and Teicher 2004; Eiland and Romeo 2013; Hueston et al. 2017).

Limbic system is a brain structure that important for controlling emotion. The component of limbic system are limbic cortex, amygdala, septal area, hypothalamus, and hippocampal formation (Rajmohan and Mohandas 2007; Palazidou 2012). The hippocampal formation consist of dentate gyrus (DG), hippocampus proper (CA1, CA2, and CA3), and subiculum (Benarroch 2013). The hippocampus is important in constructing declarative memory, encoding spatial information, regulating mood, and also important in stress response (Fa et al. 2014).

The dentate gyrus is a part of hippocampal formation that consist of 3 layers; granular layer, molecular layer, and polymorphic layer or also called as hilus (Benarroch 2013; Fa et al. 2014). Granular layer is composed of densely packed granule cells with extended dendrite throughout the molecular layer to receive an input from entorhinal cortex. The granule cells in the other end have an unmyelin axon called mossy fibers that projected to apical dendrite of the CA3 piramidal neurons (mossy fiber projection). CA3 neurons then projected to the apical dendrites of the CA1 neuron (Schaffer collateral projection) that have a bidirectional projectios from and to the basolateral amygdala (Amaral et al. 2007).

The complexity of hippocampus structure and neuronal connection makes hippocampus have neurogenesis mechanism to maintain its function and networking.

Neurogenesis is an addition of new born neuron and glia in brain. Adult neurogenesis take place in two major areas, subventricular zone (SVZ) of lateral ventricles and subgranular zone (SGZ) of DG. Neurogenesis that occur in DG are regulated by adrenal steroids hormone and have an important role in hippocampal plasticity (Cameron and Gould 1994; Mandyam 2013). Neurogenesis can be affected by many factors, including hormones, circadian-rhythm, diet, physical activity, stress, and age (Navarro-Sanchis et al. 2017).

The development of neuropsychiatric disorder in human, such as depression and anxiety disorder can caused by chronic stress (Pandey et al. 2010). Chronic stress inhibit neurogenesis and also reduces the number of neurons and DG’s volume (McEwen et al. 2016). Chronic stress that occurs during adolescence can cause a long-term effects on adult behavior and morphology of limbic system thus increasing the risk of depression (Spear 2000; Hoffmann et al. 2002; Isgor et al. 2004; Heim et al. 2008). Boldrini et al. (2013) reported, people with depression have decreased number of granular cells in DG without any reduction of neuronal progenitor cells (NPCs). There has never been a publications that directly
compares the short and long-term impact of chronic stress that occurs during adolescence.

Aim

This research aims to compare the consequences of short and long-term effect of chronic stress that occurs during adolescence on hippocampus at the behavioral level and on the morphology of hippocampal neurons in human, modeled in mice.

MATERIALS AND METHODS

Time and Location

This research was conducted from September 2017 to February 2018 in Laboratory of Animal Microtechnic and Laboratory of Animal Function and Behavior, Department of Biology, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University.

Subject and Material

Twelve of male Deutsch Democratic Yokohama (DDY) strain mice (Mus musculus albinus) (postnatal day (PND) 28; ±15 gram) were used in this research under standard laboratory conditions (temperature 22-25°C; room humidity 55-68%; 12h light-dark cycles; ad libitum access to water and food). Material used in this research are ketamine-xylazine, neutral buffered formalin (NBF) 10% fixative, ethanol (100%, 95%, 70%), paraffin, Golgi-Cox staining kit, hematoxylin-eosin (HE) staining kit, cat’s fecal and urine, hair dryer, stopwatch, Y-arm maze, elevated plus maze, light-dark transition test apparatus, rotary microtome, lamp, 44 x 36 x 14 cm³ box, histopathology preparation equipments, Olympus CX31 light microscope, and HDMI microscope camera.

Methods

Chronic Unpredictable Stress (CUS)

Chronic unpredictable stress (CUS) procedures were conducted to produce animal model of depression. One form of given stressor is an environmental demand that exceeds psychological regulatory capacity of organisms, especially stressor that occurs without anticipatory response (Lucassen et al. 2014). Mice were divided into 4 groups of experiment; non-stressed adolescent group (NS), non-stressed adult group (NL), short-term effect of stress (SS), and long-term effect of stress (LS). Mice were acclimatized for 7 days, then SS and LS groups underwent CUS procedures for 14 days, starting at PND28 (Pandey et al. 2010; Yohn and Blendy 2017). One stressor was given daily in varying times on light cycles. Detail of stressors are as follow: overnight illumination were done by exposing mice to light in 12h dark periods. Inverted light and dark cycle were performed by turned the light off during light cycle and vice versa. Hot air stream was carried out by exposing mice to hot air stream from hair dryer for 10 minutes...
(Monteiro et al. 2015) with 40 cm distance from above. Wet bedding was done by adding 50 mL of water to mice bedding for 4h. Predator odor was performed by placed cat’s feces and fur inside home cage for 10 minutes (Yohn and Blendy 2017). No bedding was done by removed mice bedding from home cage for 24h. Multiple cage changes performed by moving mice to new cage every 30 min for 4h (Barnum et al. 2012). The completed schedule can be seen at Appendix 1. Stressors were given one per day in unpredictable time and random order to prevent habituation and resemble everyday life stress (Hollis et al. 2013). After 14 days of CUS, mice underwent behavioral tests. NS and SS groups were tested during adolescence (PND43) while NL and LS groups tested during adulthood (PND77).

**Behavioral Test**

1. **Anxiety-like Behavior Measurement Using Light-Dark Transition Test**
   Light-dark transition test was used for assessing anxiety-like behavior. Light-dark box consist of two equal size of light and dark chamber divided by a partition with door (Takao and Miyakawa 2006). Light chamber is brightly illuminated whereas the dark chamber has no light (0 lux). Mice were placed in the light chamber facing the opposite wall of the door and allowed to freely explore the apparatus for 5 minutes while the behavior recorded. Frequency of light and dark chamber entries and time spent in each chambers were collected and used as a behavioral parameter of anxiety-like behavior. An entry to light or dark chamber was considered if mice placed four paws to one of the chambers, while two paws out of a given chamber considered as out.

2. **Anxiety-like Behavior Measurement Using Elevated Plus Maze (EPM)**
   Elevated plus maze (EPM) was used as second test of anxiety-like behavior. EPM apparatus consists of 4 arms (30 cm x 5 cm), 0.5 cm high walls for open arms to protect mice from falling and 15 cm high black walls for closed arms. Each arms was elevated to a height of 40 cm and placed above the table (Walf and Frye 2007). Mice were placed in the center of the maze facing one of the open arms. Mice were allowed to freely explore the maze and the behavior were recorded for 5 minutes. The number of entries into the open and closed arms were collected and used as a behavioral parameter of anxiety-like behavior. An entry to open or closed arm was considered if mice placed four paws to one of the arms, while two paws out of a given arm considered as out. The apparatus was cleaned with 70% ethanol and allowed to dry after each testing (Buccafusco 2009; Leo and Pamplona 2014).

3. **Spatial Memory Performance Measurement Using Y-alteration Test**
   Spatial memory performance was examined with the Y-arm maze. Y-maze is an apparatus that has 3 ‘Y’ shaped arms (5 x 30 cm²) and interconnected with each other at 120° angle. Maze was wiped with 70% ethanol before use. Each arms were labeled as ‘A’, ‘B’, and ‘C’ to facilitate data collection. Mice placed in the end of ‘A’ arm and recorded while freely explore the maze for 5 minutes. Arm entry occurs when all 4 paws of the mouse left the center of the maze (Miedel et al. 2017). A spontaneous alternation was used as a parameter of mice’s spatial memory. Spontaneous alternation % is calculated with the following formula.
Spontaneous alternation % = \frac{\text{# spontaneous alternation}}{\text{total number of arm entries} - 2} \times 100

**Cell Quantification**

After completion of CUS protocols and behavioral tests, mice were sacrificed under ketamine-xylazine anesthesia. The brains were quickly dissected and left hemispheres of brains were post-fixed with NBF 10% for 5 days and underwent tissue processing. After processing steps, brain tissues were embedded with paraffin. Paraffin embedded tissue were serially cut on rotary microtome, lefted on hotplate for 1 day, and stained with Hematoxylin-Eosin as described earlier (Cardiff et al. 2014) with some of modification. Detailed method can be seen in Appendix 2 and 3. Sections were observed with HDMI camera connected with light microscope. Putative neural progenitor cells (NPCs) quantification was done in subgranular zone of DG, whereas granular and apoptotic cells were counted in granular layer of DG using ImageJ.

**Dendritic Complexity Analysis**

The right hemispheres of brains were immersed in equal mixture of Solution A and B (mercuric chloride, potassium dichromate, and potassium chromate). The mixture of solutions and brains were stored in the dark bottles for 9 days at room temperature and replaced on the next day. The brains were transferred to Solution C and immersed for 4 days. Brain coronal sections (200–500 µm thick) were manually dissected and rinsed with ddH2O then placed in a mixture of Solution D, Solution E, and ddH2O. Sections were observed with HCMI camera connected with light microscope. Detailed method can be seen in Appendix 4. Morphological quantifications were conducted on DG and CA1 regions of hippocampus. Three neurons on DG and CA1 regions from each mice were traced using Affinity Designer and observed for maximum dendritic span in ImageJ. Number of intersections were analyzed using Sholl Plugin on ImageJ while total dendritic length were analyzed using NeuronJ Plugin.

**Statistical Analysis**

All data are presented as mean±SEM. One-way ANOVA followed by post hoc (Tukey) testing was used for multiple comparisons. Differences were considered significant if their probability of occurring by chance was less than 5% (p<0.05). Statistical analyses performed using SPSS software.
RESULT AND DISCUSSION

Anxiety-like Behavior Measurement

CUS procedure is conducted to produces animal model of depression, marked by an anxiety-like behavior showed in apparatus. Light-dark transition test and EPM are generating conflict situations and using rodent preferences of dark closed area (approach) and fear of high and open space (avoidance) as a measurement of anxiety-like behavior (Leo and Pamplona 2014). Anxiety mice will show a longer time spent in dark closed area. The test is needed to make sure that CUS successfully produced depressed mice.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>NS</th>
<th>SS</th>
<th>NL</th>
<th>LS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Entries Frequency (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Light</td>
<td>72.222±12.729a</td>
<td>40.152±6.658a</td>
<td>38.160±4.276a</td>
<td>35.556±1.926a</td>
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<tr>
<td>Dark</td>
<td>27.778±12.729a</td>
<td>59.848±6.658a</td>
<td>61.840±4.276a</td>
<td>64.444±1.926a</td>
</tr>
<tr>
<td>Time spent (s)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Light</td>
<td>281± 9.760c</td>
<td>84± 21.465a</td>
<td>224.333±</td>
<td>141.667±</td>
</tr>
<tr>
<td>Dark</td>
<td>17.667±9.005b</td>
<td>187±12.855a</td>
<td>53.667±</td>
<td>9.251b</td>
</tr>
</tbody>
</table>

Data are represented as mean±SEM. Mean followed by same letter in the same row are not significantly different from each other (p<0.05 ANOVA followed by Tukey post hoc test). NS: non-stressed adolescent, SS: short-term stress, NL: non-stressed adult, LS: long-term stress.

Both light and dark entries in light-dark transition test (Table 1) showed no significant light and dark chamber entries between groups (p=0.054). It means that all stressed and non-stressed groups had a same explorative behavior. However, time spent in light chamber was statistically significant (one-way ANOVA; p=0) as well as time spent on dark chamber between groups (one-way ANOVA; p=0). Non-stressed adolescent and adult have no statistically differences in both light and dark time spent. When the measures were compared within non-stressed adolescent and short-term stress, there were a significant differences on time spent in light chamber (p=0.001), indicating that non-stressed adolescent group have a higher exploration or approach behavior than stressed group (SS). A similar result shown on dark chamber (p=0), indicating that chronic stress that occurred during adolescence altered anxiety-like behavior as a short-term effect. This effect is long lasting, as seen from Table 1 that LS have a higher time spent in light chamber than SS.
Table 2  Effect of CUS on mice behavior in elevated plus maze test

<table>
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<tr>
<th>Parameters</th>
<th>Groups</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>NS</td>
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<tr>
<td>Entries frequency</td>
<td></td>
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<tr>
<td>Open</td>
<td>0±0a</td>
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<tr>
<td>Closed</td>
<td>100±0b</td>
</tr>
</tbody>
</table>

Data are represented as mean±SEM. Mean followed by same letter in the same row are not significantly different from each other (p<0.05 ANOVA followed by Tukey post hoc test). NS: non-stressed adolescent, SS: short-term stress, NL: non-stressed adult, LS: long-term stress.

Table 2 showed that both open and closed arms are given a significant differences between groups (one-way ANOVA; p=0.043). Adolescent exposure to CUS increased the frequency of open arms entries and decreased the frequency of closed arm entries in short-term stress group (Tukey post hoc test; p=0.034). This shows that non-stressed adolescent had an anxiety-like behavior by having a high avoidance behavior and CUS reduced the anxiety-like behavior. This result is contradicted with light-dark transition test, probably due to the EPM test that is done in the last order of behavioral test.

Spatial Memory Performance

Spatial ability is important to allow an individual to recall and manipulate information about spatial object in environment (Chaby et al. 2015). An overall comparison of all mice (Table 3) showed a significant correlation between groups (one-way ANOVA; p=0.007). For control groups, non-stressed adolescent and adult (p=0.014) had significant correlation. This showed that adult have a better spatial memory than adolescent at ground state. When compared to non-stressed adolescent, short-term stress group also had a better spatial memory and had no significant correlation compared to non-stressed adult (p=0.010). This fact indicated that short-term effect (SS) of CUS improved spatial memory in mice, and resembled the capability of non-stressed adult (NL) group (n.s.; p=0.992). Previous study also suggest that adolescent-stress may alter the strategy used to solved spatial challenges (Chaby et al. 2015).

Table 3  Effect of CUS on mice spatial memory performance in Y-alternation test

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Groups</th>
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<tbody>
<tr>
<td></td>
<td>NS</td>
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<tr>
<td>Spontaneous alternation</td>
<td>56.061±1.320a</td>
</tr>
</tbody>
</table>

Data are represented as mean±SEM. Mean followed by same letter in the same row are not significantly different from each other (p<0.05 ANOVA followed by Tukey post hoc test). NS: non-stressed adolescent, SS: short-term stress, NL: non-stressed adult, LS: long-term stress.

Long-term effect of CUS is statistically not significant from non-stressed adult group, indicated that CUS had no long-term impact on spatial memory. This
was probably caused by self-renewal mechanism in DG called neurogenesis that regulated by environmental factors including stress. Stress triggered neurogenesis mechanism to renewing damaged neuron and maintain brain function. This mechanism allows brain to produce an adaptive behavior (Trinchero et al. 2017).

**Cell Counting with Hematoxylin-Eosin Staining**

Cell counting was performed using HE staining. Hematoxylin give a purple stained nuclei while eosin give a red stained cytoplasm. Cell proliferation is measured by counting the number of neural progenitor cells (NPCs). NPCs observed in SGZ and have a pyramidal-like cell. Granule cells counting used to measured cell survival, marked by densely packed in granular layer with purple stained round nuclei. Apoptotic cells appeared with dark-stained pyknotic nuclei and smaller than granule cells (Hashem et al. 2010).

Figure 1  Coronal photomicrograph of hippocampal region from a hematoxylin-eosin stained brain section; (A) hippocampus region; and (B) NPCs (arrow), granule cells (thick black arrow), and apoptotic cells (white arrow).

NPCs constantly produce newborn neurons in SGZ of DG that migrate radially to granular layer (Figure 1) and known as granule cells. Granule cells (GCs) have an important role in information processing, such as during spatial learning (Benarroch 2013). Data in Table 4 displayed neither of putative NPCs
density (p=0.978), GCs density (p=0.373), nor apoptotic cells density (p=0.814) had a significant correlation between groups. This result indicated that chronic stress have no effect on reducing proliferation, cell maturing, and cell survival in DG. In contrast, previous studies showed that chronic stress reduces the proliferation, survival (number of GCs), and also dysregulate apoptosis in the DG (Castilla-Ortega et al. 2011). Boldrini et al. (2013) also reported that depressed patients have a fewer GCs in DG without fewer NPCs.

Hippocampal plasticity allows brain to produces new GCs by neurogenesis mechanism and promotes synaptic remodeling (Trinchero et al. 2017). Stress can inhibit neurogenesis and survival of newborn cells that also increases apoptosis of progenitor cells (Krugers et al. 2010; Lucassen et al. 2010). Heine et al. 2014 stated that the effect of chronic unpredictable stress showed a reversible of reducted proliferation and reversible in increases number of apoptotic cells. While Y-alternation test showed a better spatial learning on short-term effect of CUS, cell counting in contrast did not reveal a short and long-term effect of CUS on spatial learning. This contrast finding is most likely caused by inability of HE staining to fully detected a high proliferation rate in adolescent mice. In this case, Bromodeoxyuridine (BrdU) immunohistochemistry preferably to be used for monitoring cell proliferation (Taupin 2007). There is also a possibility that the enhancement of spatial memory are not affected by NPCs and proliferation rate, but by another mechanism or part of brain that also contributes in spatial learning and memory.

Table 4  Effect of CUS on cell proliferation, survival, and apoptosis

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Groups</th>
<th>NS</th>
<th>SS</th>
<th>NL</th>
<th>LS</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCs</td>
<td></td>
<td>62.605±14.557</td>
<td>64.656±13.953</td>
<td>48.832±2.211</td>
<td>78.399±8.972</td>
</tr>
<tr>
<td>ACs</td>
<td></td>
<td>10.007±1.551</td>
<td>10.531±3.138</td>
<td>8.155±0.438</td>
<td>8.946±1.392</td>
</tr>
</tbody>
</table>

Data are represented as mean±SEM (p<0.05 ANOVA followed by Tukey post hoc test). NS: non-stressed adolescent, SS: stress adolescent, NL: non-stressed adult, LS: stress adult. NPCs: neural progenitor cells, GCs: granular cells, ACs: apoptotic cells.

Dendritic Complexity Analysis

Golgi-Cox staining is used to study the morphology of neuron, performed in hippocampal region (Ganesh et al. 2014). Wu et al. (2015) stated, there are 2 types of principal neuronal in hippocampal formation. Pyramidal cells of CA1 have a biconal shape with thick apical dendrite and basal dendrite in opposite directions of soma. CA1 is the primary output of the hippocampus that integrate information from two parallel paths in the entorhinal cortex (EC); a direct EC-CA1 projection and an indirect EC projection through CA3 and dentate gyrus. CA1 have been reported to have a functional role in contextual memory retrieval (Ji and Maren 2008).
The second type of principal neuron is dentate gyrus cells that morphologically have a different dendritic arbors. Newborn neurons from SGZ undergo short radial migration to granular layer and differentiate into immature neurons. This immature dentate gyrus cells (DGCs) have an apical dendrites that faced the molecular layer of DG and involved in information processing (Bennaroch 2013).

Figure 2  Coronal photomicrograph of hippocampal region from a Golgi-Cox stained brain section.

Figure 3  Example of neuron morphological analysis; (A) number of intersections measurement using ImageJ Sholl plug-in; (B) maximum dendritic span measurement using ImageJ Sholl; (C) total dendritic length measurement using NeuronJ plug-in.
Dendritic Complexity Analysis in DG Region

Table 5  Effect of CUS on dendritic morphology of dentate gyrus neuron

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intersections</td>
<td>NS</td>
</tr>
<tr>
<td>Maximum dendritic span</td>
<td>62.605±14.557</td>
</tr>
<tr>
<td>Total dendritic length</td>
<td>10.007±1.551</td>
</tr>
</tbody>
</table>

Data are represented as mean±SEM. Mean followed by same letter in the same row are not significantly different from each other (p<0.005 ANOVA followed by Tukey post hoc test).


Figure 4  Traced dentate gyrus neurons; scale bar: 100 µm.

The result of Sholl analysis (Table 5) showed a significant mean number of intersections between groups (one way ANOVA; p=0). Complexity of intersections changes were showed from non-stressed adolescent and adult (Figure 4). Non-stressed adult displayed a higher number of intersections (Tukey post hoc test; p=0). CUS turn out did not changing the number of intersection for both adolescence and adult. However, there was no significant short and long-term effect of CUS between groups on maximum dendritic span (one-way ANOVA; p=0.341) and total dendritic length (one-way ANOVA; p=0.425).
Dendritic Complexity Analysis in CA1 Region

Figure 5  Traced apical dendrites of CA1 pyramidal neurons; scale bar: 100 µm.

The intersection of CA1 apical dendrites (Table 5) were significantly different between groups (one-way ANOVA; p=0). Both non-stressed group (NS and NL) showed a similar low numbers of intersection. CUS showed a short-term effect in number of intersections (p=0), NL and LS (p=0.001). In contrast, neither of the total dendritic length (one-way ANOVA; p=0.108) and maximum dendritic span (p=0.100) showed a significantly different although both graphics showed stressed group (SS and LS) have a higher degrees of dendritic span and longer total dendritic length.

Similar to the observation in apical dendrites of CA1, the intersections of CA1 basal dendrites (Table 5) were significantly different between groups (one-way ANOVA; p=0). Non-stressed adolescent and adult did not have a significant differences in CA1 basal dendrites intersections number but short-term effect CUS caused intersections of CA1 basal dendrites to be higher in numbers as seen in Figure 6 (p=0.019). The long-term effect also shown a significant changes in number of intersections (p=0.007). Both maximum dendritic span (p=0.470) and total dendritic length (p=0.4528) revealed no significant differences between groups. Wooley et al. (1990) and Sousa et al. (2000) in contrast have found that

### Table 6  Effect of CUS on dendritic morphology of CA1 pyramidal neuron

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Groups</th>
</tr>
</thead>
<tbody>
<tr>
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<td>NS</td>
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<tr>
<td>Apical dendrites</td>
<td></td>
</tr>
<tr>
<td>Intersections</td>
<td>1.722±0.180a</td>
</tr>
<tr>
<td>Maximum dendritic span</td>
<td>53.551±18.975</td>
</tr>
<tr>
<td>Total dendritic length</td>
<td>485.207±36.202</td>
</tr>
<tr>
<td>Basal dendrites</td>
<td></td>
</tr>
<tr>
<td>Intersections</td>
<td>2.037±0.190a</td>
</tr>
<tr>
<td>Total dendritic length</td>
<td>585.192±393.611</td>
</tr>
</tbody>
</table>

Data are represented as mean±SEM. Mean followed by same letter in the same row are not significantly different from each other (p<0.005 ANOVA followed by Tukey post hoc test).

A high level of glucocorticoid or chronic stress did not change both of apical and basal dendritic length of CA1 pyramidal neurons.

Figure 6. Traced basal dendrites of CA1 pyramidal neurons; scale bar: 100 µm.

CA1 is the major output of the hippocampus that contributes in recognizing the novelty or familiarity of an object or context (Nakazawa et al. 2004; Barrientos and Tiznado 2016). The increased complexity of CA1 dendrites in both SS and LS groups showed that CUS may improve CA1 ability to retrieve contextual memory.

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

Chronic unpredictable stress produces a depressed-like mice. Y-alternation test showed that CUS increases spatial learning in adolescent to the same level of adult capability. CUS have no short and long-term effect on proliferation, cell maturing, and cell survival in DG. Similar result also seen in dendritic complexity of DG that not significant different between groups. It is remarkable that both short and long-term effect of CUS improves encoding and retrieval of contextual memory by changing the morphology and complexity of CA1 pyramidal neuron.

Recommendations

BrdU immunohistochemistry should be considered to replacing hematoxylin-eosin staining method for monitoring proliferation rate on hippocampus. Further research is needed to determine the effect of chronic unpredictable stress on CA1 pyramidal neuron and its function.
REFERENCES


## APPENDIXES

### Appendix 1  Schedule for the chronic unpredictable stress procedure

<table>
<thead>
<tr>
<th>Week</th>
<th>Days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Mon</strong></td>
</tr>
<tr>
<td>1</td>
<td>Hot air stream 2h</td>
</tr>
<tr>
<td>2</td>
<td>Hot air stream 12h</td>
</tr>
</tbody>
</table>
Appendix 2 Preparation of brain tissue (processing and paraffin embedding method)

Granular cells, NPCs, and apoptotic cells quantification was done by preparing brain photomicrograph slides as previously described with modifications (Alifah 2017). The steps of brain photomicrograph making are as follow:

1. Mice brains were isolated and sagittaly dissected.
2. Left hemispheres of brains were placed inside tissue cassette and fixed with neutral buffered formaline (BNF) 10% for 5 days at room temperature.
3. After 5 days of fixation, brains were moved to ethanol 70% for at least 24 hours.
4. Brains were moved to 80% ethanol, 90% ethanol, and absolute ethanol for 1 hour each and moved to alcohol:xylol (1:1) for 1 hour.
5. Brains were dipped into one changes of xylol (act as a clearing agent) for 30 minutes each in room temperature and once more xylol for 40 minutes in paraffin oven (60ºC).
6. Brains were dipped into two changes of paraffin for each 45 minutes for paraffin infiltration.
7. Brain samples were transferred to mold and filled with hot paraffin.

Appendix 3 Hematoxylin-eosin staining method

Hematoxylin and eosin stain is a standard stain for microscopic examination on histology. After preparation steps, brain samples were undergo staining steps as described by Cardiff et al. (2014) with some of modifications as follow:

1. Paraffin embedded mice brains were sectioned using rotary microtome with 5 µm thick.
2. The sectioned slides were placed on paraffin stretcher for at least 12 hours.
3. Slides were hydrated as follows.
   Dipped in absolute ethanol for 6 minutes.
   Transfer to 95% ethanol and 70% for 2 minutes each.
4. After hydrated, slides were washed with running tap water in room temperature for 2 minutes.
5. Slides were stained with hematoxylin solution for 3 minutes.
6. Remains hematoxylin in slides were rinsed with running tap water in room temperature for 2 minutes.
7. Slides were stained with eosin solution for 2 minutes.
8. Samples were dipped in ethanol 95 % 20 times and transferred to ethanol 95% for 3 minutes, and absolute ethanol 100% for 4 minutes.
9. Samples were cleared with xylol for 10-15 minutes.
10. A drop of entellan was added over the tissue followed with cover glass.
Appendix 4  Golgi-Cox staining method

Golgi-Cox is a staining method to examining neuron cell morphology. The brains were processed for Golgi-Cox staining according to FD Rapid GolgiStain™ Kit manufacturer’s instructions with some modifications as follows:

1. Right hemisphere of brains were dipped into an equal mixture of Solution A and B that prepared 24 hours before use, unstirred, and protected from light. Solution A and B containing mercuric chloride, potassium dichromate, and potassium chromate.

2. Replaced Solution A and B on the next day.

3. After 9 days, brain tissues were transferred into Solution C for 3 days, and the solution were replaced on the next day.

4. Brains were moved to styrofoam with a few drops of Solution C to prevent brains from drying, then manually cut with 200~500 µm thickness.

5. Brain sections were moved to glass slides and washed with double destilled water (ddH2O) 2 times, 4 minutes each.

6. Mixed of 1 part Solution D, 1 part Solution E, and 1 part ddH2O was made and dropped to the brain sections.

7. Photomicrograph of mouse brain tissue using Golgi-Cox staining can be analyzed using light microscope.
CURRICULUM VITAE

The author was born on June 12th, 1996 in Jakarta as the first child of three from parents Hendry Susanto and Suliani. In 2014, author graduated from SMAK Ketapang III and joined Department of Biology, Bogor Agricultural University through SBMPTN.

By end of 4th semesters, author conducted a field study at Ujung Kulon National Park with research title “Pasang Surut Kehidupan di Daerah Pasang Surut” and field work by the end of 6th semesters in Turtle Conservation and Education Center (TCEC) Serangan, Bali with research title “Relokasi Telur dan Pelepasliaran Tukik di Turtle Conservation and Education Center, Serangan, Denpasar, Bali”. In 2016, author gained an achievement as the Third Winner of Pekan Ilmiah Mahasiswa Nasional (PIMNAS) 29 by Ristekdikti on poster category.

Author also contributed in Himpunan Mahasiswa Biologi (Himabio IPB) for two periods in 2015-2016 and 2016-2017 and was active in many committees as head of publication, decoration, and documentation division of MIPA Khitanan Massal 2016 held by BEM FMIPA, Retreat PMK 2016, and BIONIC national seminar and workshop 2016. In 2017-2018, author became a laboratory assistant for Histology, Vertebrate, and Basic Biology.