Effect of chronic paracetamol treatment on the alteration of learning and memory in rats

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ผลของการได้รับยาพาราเซตามอลอย่างเรื้อรังต่อการเปลี่ยนแปลงการเรียนรู้และความจำในหนูแรท

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ลัดดาวรรณ ละเลิศ : ผลของการได้รับยาพาราเซตามอลอย่างเรื้อรังต่อการเปลี่ยนแปลงการเรียนรู้และความจำในหนูแรท. (Effect of chronic paracetamol treatment on the alteration of learning and memory in rats) อ.ที่ปรึกษาหลัก : ผศ. ดร.ศุภางค์ มณีศรี เลอกรองด์, อ.ที่ปรึกษาร่วม : ศ. นพ.อนันต์ ศรีเกียรติขจร,รศ. นพ.สมพล สงวนรังศิริกุล

พาราเซตามอลเป็นยาที่ใช้กันอย่างกว้างขวางเพื่อบรรเทาอาการปวดและลดไข้ แม้ว่ายาชนิดนี้ได้รับการยอมรับว่ามีความปลอดภัย แต่ปัจจุบันหลายงานวิจัยได้รายงานว่าการใช้ยาพาราเซตามอลสามารถก่อให้เกิดการเปลี่ยนแปลงของ neurobehaviors ต่างๆได้ อย่างไรก็ดีผลกระทบที่แท้จริงของการได้รับยาพาราเซตามอลต่อการทำหน้าที่ด้านความคิดความเข้าใจ (cognitive function) และกลไกที่เกี่ยวข้องต่อการเกิดผลกระทบดังกล่าวยังไม่เป็นที่ทราบอย่างแน่ชัด การศึกษานี้จึงมีวัตถุประสงค์เพื่อศึกษาผลกระทบของการได้รับยาพาราเซตามอลต่อการเปลี่ยนแปลงการเรียนรู้และความจำและศึกษากลไกของการเกิดผลกระทบจากการได้รับยาพาราเซตามอล ในการศึกษาวิจัยนี้ ยาพาราเซตามอลขนาด 200 มิลิกรัมต่อกิโลกรัมน้ำหนักตัวถูกป้อนทางปากให้กับหนูแรทเพศผู้สายพันธุ์วิสต้าร์เพียงหนึ่งครั้งสำหรับกลุ่มที่ได้รับยาอย่างเฉียบพลัน และได้รับหนึ่งครั้งต่อวันต่อเนื่องเป็นเวลา 15 และ 30 วัน ในกลุ่มที่ได้รับยาแบบกึ่งเรื้อรังและเรื้อรังตามลำดับ การเปลี่ยนแปลงของความจำชนิด recognition และ spatial memory ของหนูแรททุกกลุ่มถูกทดสอบด้วยเทคนิค novel object recognition (NOR) และ Morris water maze (MWM) ตามลำดับ หลังจากนั้นทำการตรวจสอบการเปลี่ยนแปลงโครงสร้างและโปรตีนของ synapses (SYP และ PSD-95) ที่สัมพันธ์กับกระบวนการการเรียนรู้และความจำ และทำการวิเคราะห์การแสดงออกของโปรตีน brain-derived neurotrophic factor (BDNF) ในสมองบริเวณ frontal cortex และ hippocampus ของหนูแรททุกกลุ่ม นอกจากนั้นเพื่อศึกษาความเกี่ยวข้องของ oxidative stress ต่อการเปลี่ยนแปลงการเรียนรู้และความจำจากการได้รับยาพาราเซตามอล การศึกษาในครั้งนี้จึงทำการวิเคราะห์การเปลี่ยนแปลงของ oxidative stress markers ได้แก่ protein carbonyl oxidation (PCO) ระดับ glutathione (GSH) และการแสดงออกของโปรตีน nuclear factor erythroid-2-related factor 2 (Nrf2) ในสมองบริเวณ frontal cortex และ hippocampus ผลการศึกษาวิจัยพบว่าการได้รับยาพาราเซตามอลอย่างเฉียบพลันไม่มีผลต่อการเปลี่ยนแปลงการเรียนรู้และความจำเมื่อเปรียบเทียบกับกลุ่มควบคุมและไม่พบการเปลี่ยนแปลงใดๆของ synapses การแสดงออกของโปรตีน BDNF และ oxidative stress markers ในกลุ่มที่ได้รับยาแบบเฉียบพลัน อย่างไรก็ตามผลการศึกษาพบว่าการได้รับยาพาราเซตามอลเป็นเวลายาวนานส่งผลให้มีการลดลงของ NOR และ MWM performance ในหนูแรท ผลการศึกษาในกลุ่มที่ได้รับยาแบบกึ่งเรื้อรังได้แสดงให้เห็นถึงความผิดปกติของ synaptic interfaces (ความสั้นลงของ active zone และความกว้างขึ้นของ synaptic cleft) และการลดลงของโปรตีน SYP และ PSD-95 ในสมองบริเวณ hippocampus ของกลุ่มที่ได้รับยาพาราเซตามอล ซึ่งการเปลี่ยนแปลงเหล่านี้ถูกพบควบคู่ไปกับการลดลงของโปรตีน BDNF อีกด้วย และเป็นที่น่าสนใจว่าความผิดของ synapses ได้ถูกแสดงให้เห็นอย่างชัดเจนในกลุ่มที่ได้รับยาแบบเรื้อรัง ซึ่งนอกจากการเปลี่ยนแปลงของ synaptic interfaces (ความสั้นลงของ active zone และความกว้างขึ้นของ synaptic cleft) แล้ว ยังพบการลดลงของจำนวน synapses ทั้งในสมองบริเวณ frontal cortex และ hippocampus ในกลุ่มที่ได้รับยาพาราเซตามอล และพบว่าระดับการแสดงออกของโปรตีน BDNF ในสมองบริเวณ frontal cortex และ hippocampus ได้ลดลงในกลุ่มนี้ด้วยเช่นเดียวกัน นอกจากนี้ผลการศึกษายังแสดงให้เห็นว่ามีการเปลี่ยนแปลงของ oxidative stress markers ที่บ่งชี้ถึงการเกิดภาวะ oxidative stress (การเพิ่มขึ้นของโปรตีน Nrf2 และระดับ PCO รวมถึงการลดลงของ GSH) ในสมองบริเวณ hippocampus ในหนูกลุ่มที่ได้รับยาพาราเซตามอลแบบเรื้องรังอีกด้วย จากผลจากการศึกษาทั้งหมดสามารถสรุปได้ว่าการได้รับยาพาราเซตามอลเป็นเวลายาวนานสามารถเหนี่ยวนำให้เกิดความบกพร่องของการเรียนรู้และความจำ ซึ่งการเกิดภาวะ oxidative stress ในสมองภายหลังจากการได้รับยาพาราเซตามอลอย่างยาวนานน่าจะมีส่วนเกี่ยวข้องกับการเปลี่ยนแปลงการแสดงออกของโปรตีน BDNF และ synaptic integrity ในสมองบริเวณ frontal cortex และ hippocampus ซึ่งสามารถนำไปสู่ความผิดปกติของการเรียนรู้และความจำได้ในที่สุด

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Paracetamol (acetaminophen: APAP) is widely used for treatment of pain and fever. Although APAP is well accepted as a safe drug, several studies have recently demonstrated that treatment with this drug can lead to an alteration of several neurobehaviors. However, the exact impact of the APAP treatment and the mechanisms underlying those effects are still largely unknown. The present study aimed to investigate the effect of APAP treatment on the alteration of learning and memory and the possible mechanism underlying deleterious effects induced by APAP treatment. In this study, APAP at the dose of 200 mg/kg bw was orally fed to adult male Wistar rats through either acute (0 days), sub-chronic (15 days) or chronic (30 days) treatment regimens. The recognition and spatial memory in all experimental groups were assessed by using the novel object recognition (NOR) and Morris water maze (MWM) test, respectively. The changing of synaptic ultrastructure and proteins, synaptophysin (SYP) and postsynaptic density-95 (PSD-95), related with learning and memory were as well monitored in both the frontal cortex and hippocampus. Moreover, the expression of brain-derived neurotrophic factor (BDNF) was examined in all experimental groups. To investigate the involvement of oxidative stress on the alteration of learning and memory following APAP treatment, the oxidative stress markers including protein carbonyl oxidation (PCO), glutathione (GSH) level and nuclear factor erythroid-2-related factor 2 (Nrf2) were evaluated in the frontal cortex and hippocampus obtained from all experimental groups. The results demonstrated that, as compared with control, acute treatment with APAP had no any effect on learning and memory abilities. The alteration of synapses, BDNF protein expression and oxidative stress markers were not observed in the rats with 0-day APAP treatment. However, the results obtained from the rats treated with APAP for longer periods (sub and chronic treatments) demonstrated a reduction of NOR and MWM performance. The results obtained from the experiment with sub-chronic treatment showed an abnormality of synaptic interfaces (shortening in the active zone and widening of the synaptic cleft) as well as decrements of both SYP and PSD-95 in the hippocampus of the rats with APAP treatment. These alterations were observed in parallel with a down-regulation of hippocampal BDNF protein in those rats. Interestingly, an abnormality of the synapses was clearly demonstrated in the rats with chronic APAP treatment. In addition to the changing of synaptic interfaces (shortening of the active zone and widening of the synaptic cleft), the numbers of synapses in both the frontal cortex and hippocampus were decreased in rats with chronic APAP treatment. The reduction of both frontal cortical and hippocampal BDNF levels were also detected in these rats. Moreover, the alteration of oxidative stress markers indicating an increase of oxidative stress formation (up-regulation of Nrf2 protein, increment of PCO level, and depletion of GSH) were clearly demonstrated in the hippocampus obtained from chronic APAP treated group. Based on the results obtained from this study, we suggest that treatment with APAP for long period can induce an impairment of learning and memory. An increase of oxidative stress in the brain following chronic APAP treatment is possibly participated in the alteration of BDNF protein and synaptic integrity in the frontal cortex and hippocampus which can finally lead to an abnormality of learning and memory performance.

Field of Study: Medical Sciences
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LIST OF ABBREVIATIONs

AD	Alzheimer's Disease
ADHD/HKD	Attention Deficit Hyperactivity Disorder/Hyperkinetic Disorder
ALP	Alkaline Phosphatase
ALT	Aspartate Aminotransferase
AM404	Acidamide N-Arachidonoyl-Phenolamine
AMPA	α-Amino-3-Hydroxy-5-Methylisoxazole-4-Propionic Acid
Amy	Amygdala
APAP	Paracetamol (or Acetaminophen)
AREs	Adenylate-Uridylate-Rich Elements
AST	Alanine Aminotransferase
BBB	Blood-Brain Barrier
BDNF	Brain-Derived Neurotrophic Factor
CA	Cornu Ammonis
CBs	Cannabinoids
CNS	Central Nervous System

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COPD	Chronic Obstructive Pulmonary Disease
COX	Cyclooxy-Genase
CSF	Cerebrospinal Fluid
CSD	Cortical Spreading Depression
CYPs	Cytochromes P450
DG	Dentate Gyrus
DNPH	2,4-Dinitro-Phenylhydrazine
EC	Entorhinal Cortex
ER	Endoplasmic Reticulum
FDA	Food and Drug Administration
γGCS	γ-Glutamylcysteine Synthetase
GCLC	Glutamate Cysteine Ligase (GCL)-Catalytic Subunit
GCLM	Glutamate Cysteine Ligase (GCL)-Modifier Subunit
GPX	Glutathione Peroxidase
GSH	Glutathione
GSR	Glutathione Reductase
GSS	Glutathione Synthetase
GSSG	Glutathione Disulfide

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HO-1	Hemoxygenase
H2O2	Hydrogen Peroxide
5-HT	5-Hydroxytryptamine (or Serotonin)
KEAP1	Kelchlike ECH Associated Protein 1
LTP	Long-Term Potentiation
mPFC	Medial Prefrontal Cortex
MWM	Morris Water Maze
NAcc	Nucleus Accumbens
NAPQI	n-acetyl-p-benzoquinoneimine
NF-kB	Nuclear Factor-kappa B
NMDA	N-Methyl-D-Aspartate
NQO1	NAD(P)H Quinone Oxidoreductase 1
NOR	Novel Object Recognition
Nrf2	Nuclear Factor Erythroid 2–Related Factor 2
NT	Neurotransmitter
PBS	Phosphate Buffer Saline
PCO	Protein Carbonyl Oxidation
PD	Parkinson's Disease

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PGH2	Prostaglandins
PGHS	Prostaglandin H2 Synthase
PSD	Post-Synaptic Density
PSD-95	Postsynaptic Density-95
RE	Thalamic Nucleus Reuniens
ROS	Reactive Oxygen Species
SEM	Standard Error of Mean
SNARE	Soluble N-Ethylmaleimide-Sensitive Factor Activating Protein Receptor
SOD	Superoxide Dismutase
SUB	Subiculum
SR	Stratum Radiatum
SYP	Synaptophysin
TCA	Trichloroacetic Acid
TEM	Transmission Electron Microscope
TrkB	Tropomyosin Receptor Kinase B
TRPA1	Transient Receptor Potential Ankyrin-1 Channel
TRPV1	Vanilloid Subtype 1 Receptor

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CHAPTER I

INTRODUCTION

1.1 Background and rationale

Paracetamol (Acetaminophen: APAP) is an antipyretic and analgesic drug used worldwide. Due to its properties with high availability, inexpensiveness, and minimal side-effects, there is a high possibility that the people who have chronic pain will select this drug for long term treatment. Even though APAP is widely used for over 100 years, the mechanism of action is still not fully clarified. The numbers of studies have proposed several mechanisms underlying its analgesic and antipyretic effect of this drug, including modulation of central serotonergic system, inactivation of prostaglandin H2 synthetase, activation of nitric oxide production, and stimulation of cannabinoid receptor [1-3].

Numerous evidences have demonstrated the beneficial effects of this drug treatment in several pathological conditions. It has previously revealed that APAP treatment could attenuate the neuronal cell death induced by amyloid β-peptide. The antioxidant and anti-transcriptional properties of APAP were explained as a mechanism underlying the protective effect of this drug [4]. The antioxidant and anti-inflammatory activities of APAP have been confirmed by the results observed by Tripathy and Grammas, which demonstrated that pretreatment with APAP could prevent oxidative stress-induced neuronal and endothelial cell damage [5, 6].

However, during the last decade, several studies have demonstrated the unwanted effects of APAP in several systems. In 2000, Dimova and co-workers have demonstrated that treatment with APAP could decrease cell viability in rat type II pneumocytes and alveolar macrophages. These alterations were accompanied by the decrement of glutathione (GSH) levels. This study has suggested that the

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cytotoxic effect of APAP is associated with the bioactivation of cytochrome P450 (CYPs) enzyme [7]. Moreover, the adverse effect of this drug treatment on the respiratory system was also revealed by Nassini and co-workers. They found that exposure to APAP could produce elevated proinflammatory neuropeptides in the airway sensory nerves. These results have concluded that APAP may contribute to the risk of chronic obstructive pulmonary disease and asthma, and these effects might be mediated by the generation of a reactive metabolite, n-acetyl-p-benzoquinoneimine (NAPQI) following APAP treatment [8].

Interestingly, the non-beneficial effect of this drug treatment has been reported in the central nervous system (CNS) as well. The study by Posadas and colleagues has revealed that APAP at the doses below those required to produce hepatotoxicity could produce the neurotoxic effect both *in vitro* and *in vivo* experiments [9]. The increase in the toxic metabolite of APAP converted by enzyme CYP2E1, NAPQI, has been proposed as the mechanisms underlying those harmful effects of this drug [9, 10]. It has previously been reported that NAPQI itself has an extremely toxic effect. It can directly bind to cellular macromolecules which lead to cell injury and dysfunction [11]. Normally, NAPQI can be detoxified by interacting with exist GSH in the cells. However, in case of high production of NAPQI, it can result in a decrement of GSH level which finally can induce an oxidative stress [12, 13]. Regarding NAPQI formation, it is known that the enzyme CYP2E1 is wildly distributed in the brain. The expression of this enzyme has been as well found in the frontal cortex and hippocampus [14], which are the brain regions associated with learning and memory [15, 16]. Therefore, recently, the effect of APAP treatment on learning and memory deficit has recently been focused.

The study by Ishida et al revealed that short-term treatment with a high-dose of APAP (302.3 mg/kg) could induce an impairment of learning and memory in

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mice [17]. In 2013, the Norwegian sibling-control study demonstrated that the children who exposed chronically to prenatal APAP had poorer gross motor development and higher risk for adverse psychomotor and behavioral outcomes such as attention deficit hyperactivity disorder/hyperkinetic disorder (ADHD/HKD) [18]. The effect of APAP exposure on learning and memory was confirmed by recent evidence obtained from an animal experiment which demonstrated that neonatal APAP treatment affected the spatial learning in adulthood. The fluctuation of brain-derived neurotrophic factor (BDNF) level in the frontal cortex and parietal cortex were assumed to be involved in these alterations [19].

Interestingly, the effect of APAP treatment on the hippocampus, the brain region responsible for the learning and memory, has been revealed. In 2011, Fakunle and colleagues demonstrated that chronic exposure to APAP could produce neuronal damage in the hippocampus and this effect was more severe in combination with alcohol consumption [20]. Later, in 2013, Chantong et al revealed that chronic treatment with APAP alone could elevate pro-inflammatory cytokines in the hippocampus [21].

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Even though several adverse effects of APAP treatment on learning and memory have been demonstrated, the mechanisms underlying those effects have not been clarified yet. Therefore, the effects of APAP treatment (the therapeutic dose) in three different durations (0, 15 and 30 days) on the alteration of learning and memory were investigated in this study. The recognition and spatial memory behaviors were monitored by the novel object recognition (NOR) and Morris water maze (MWM) tests, respectively. Besides, the alteration of synaptic structures which involves in the learning and memory including the number of synapses, the length of the synaptic active zone, the width of synaptic cleft, the thickness of postsynaptic density (PSD), and the synaptic interfacial curvature in both the hippocampus and

medial prefrontal cortex (mPFC) were investigated in rats with APAP treatment compared to those of the control group. Moreover, the alteration of proteins related to synaptic plasticity (synaptophysin and postsynaptic density-95) were as well detected in all animals.

It is known that the oxidative stress and BDNF are involved in learning and memory processes [22-26], therefore an expression of BDNF was monitored in the hippocampus and frontal cortex in all animals. In addition, the oxidative stress markers including the levels of GSH, Nuclear factor erythroid 2–related factor 2 (Nrf2) protein expression, and the content of protein carbonyl oxidation (PCO) were also evaluated in both brain regions.

The results obtained from this study are expected to provide a shred of strong evidence confirming the effect of chronic APAP treatment on learning and memory. This knowledge can, at least, be used as a possible guideline for using APAP as a chronic treatment.

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1.2 Keywords

Paracetamol, Chronic treatment, Learning and memory, Brain-derived neurotropic factor, Synaptophysin, Postsynaptic density-95, Synaptic structural plasticity, Oxidative stress

1.3 Research design

Animal experiment

1.4 Research questions

1.4.1 Does chronic APAP treatment alter learning and memory ability?

1.4.2 Does chronic APAP treatment induce the alteration of synaptic structure and synaptic proteins in the hippocampus and frontal cortex?

1.4.3 Does chronic APAP treatment cause an alteration of BDNF protein expression in the hippocampus and frontal cortex?

1.4.4 Does chronic APAP treatment produce oxidative stress in the hippocampus and frontal cortex?

1.4.5 Does chronic APAP treatment induce the alteration of Nrf2 protein expression in the hippocampus and frontal cortex?

1.5 Objectives

Major objective

To clarify the effect of chronic APAP treatment on the alterations of learning and memory in rats

Minor objectives

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1. To investigate the effect of chronic APAP treatment on the alteration of synaptic structures in the hippocampus and frontal cortex.

2. To determine the effect of chronic APAP treatment on the expression of synaptic proteins including SYP and PSD-95 in the hippocampus and frontal cortex.

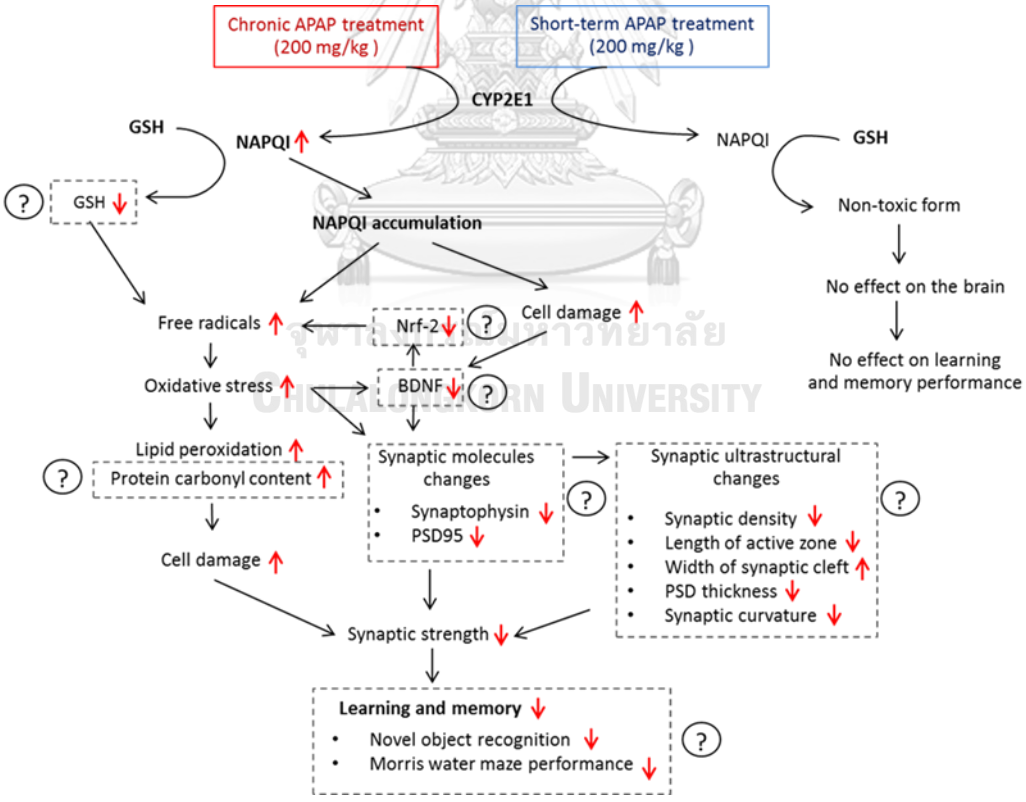
3. To evaluate the effect of chronic APAP treatment on the expression of BDNF in the hippocampus and frontal cortex.

4. To investigate the effect of chronic APAP treatment on the oxidative stress (GSH level, Nrf2 protein expression, and the content of protein carbonyl oxidation) in the hippocampus and frontal cortex.

1.6 Hypothesis

Chronic APAP treatment will result in the elevated NAPQI formation and the depletion of GSH in the brain. The accumulation of NAPQI and the increasing of oxidative stress from GSH depletion can result in decreased BDNF expression. The alteration of BDNF protein will lead to a decrement of synaptic proteins and abnormality of synaptic structural plasticity, which finally cause the disturbance of learning and memory.

1.7 Conceptual framework



CHAPTER II

REVIEW LITERATURES

2.1 Cognitive function, Hippocampus and Frontal cortex

Cognitive function is the mental action or process which is explained as the ability to know including learning, memory, reasoning and executive function. Learning is the accomplishment of new information and leads to the behavior adaptation which is resulted from individual experience, while the memory is the preservation of information which is produced by the learning processes [27]. The hippocampus and frontal cortex are the key brains which are implicated for processing learning and memory. The critical role of the hippocampus is long-term memory consolidation. Hippocampus consolidates the original steps of long-term storage and then slowly transforms the information into storage system in neocortex [16], while the medial prefrontal cortex (mPFC) serves the function for mediating the decision which is involved in the retrieval of long-term memory that associated with context, locations, events, and behavioral adaptation [15].

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The hippocampus has many subfields which are responsible for the memory consolidation as follows: Cornu Ammonis (CA) 1 to CA3 and the dentate gyrus (DG) [28]. The connection between hippocampus and subiculum is called the hippocampal formation as shown in Figure 1. The major input pathway to the hippocampus is the perforant pathway which originated from the entorhinal cortex and then projects to granular neurons in dentate gyrus subfield. The neuronal projection of granular cell in dentate gyrus named as mossy fibers terminates on the pyramidal cells in the CA3 region. The axons of the cells in CA3 subfield further project to the CA1 neurons through schaffer collaterals pathway and axons of

neurons in CA1 then project to subiculum which sends the neuronal signal back to the entorhinal cortex [16].

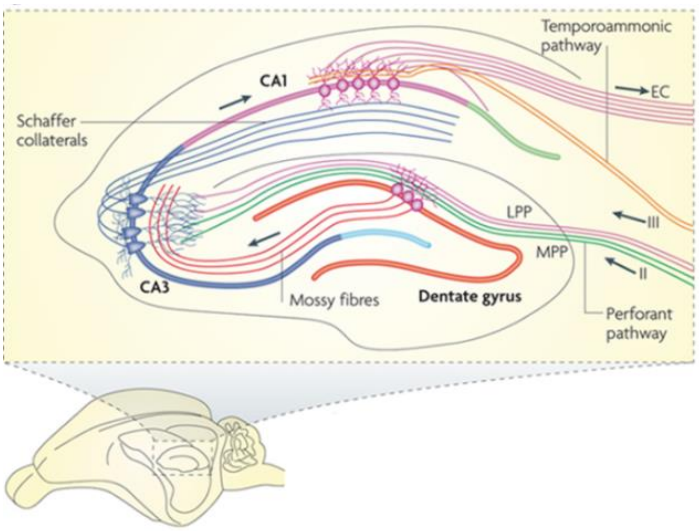

Figure 1 Diagram illustrating the hippocampal formation [29]

The link between hippocampus and prefrontal cortex is believed to implicate in the cognitive process. The major outputs from the hippocampal CA1 region and the subiculum (SUB) directly project to the medial prefrontal cortex (mPFC), but there is no the direct projection from the mPFC back to the hippocampus. The mPFC projects to both medial and lateral entorhinal cortex (EC) which are the majors input pathway to the hippocampus [30, 31]. Moreover, it has recently been proposed that the thalamic nucleus reuniens (RE) and amygdala (Amy) have mutual connections with both the mPFC and hippocampus. Furthermore, the nucleus accumbens (NAcc) has also been reported to be the interconnection among the neuronal circuits by receiving the input from the mPFC, hippocampus, RE, and Amy [32]. The connection between hippocampus and prefrontal cortex is illustrated in Figure 2.

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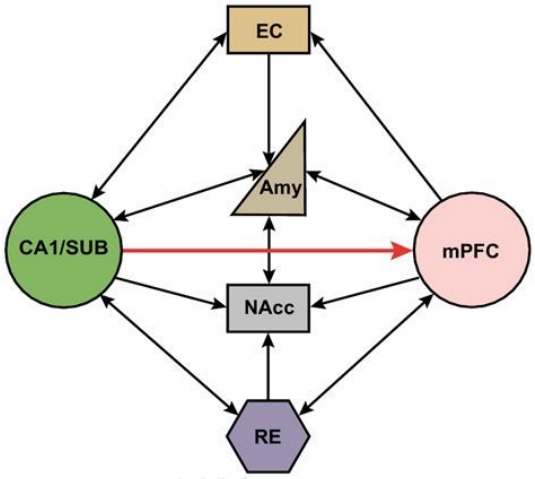

Figure 2 The connection between the hippocampus and prefrontal cortex [32]

2.2 Synaptic plasticity-associated learning and memory process

In the nervous system, a synapse is a specialized structure permitting a neuron communicates to other neurons or different cell types through their electrical or chemical signal [33]. This structure has an adaptable property for serving a physiological basis of synaptic functional plasticity [34, 35]. A large body of evidence has proposed that long-term potentiation (LTP), a typical phenomenon of synaptic functional plasticity, is the mechanism underlying learning and memory consolidation [22, 36].

In addition, the synaptic structural plasticity is also found to be directly associated with LTP induction and it is considered to be implicated with the learning and memory processes [34]. The synaptic structural plasticity is comprised in many structural aspects including the alteration of the number and size of synapses, the length of the active zone, the width of the synaptic cleft, the thickness of postsynaptic density (PSD), as well as the synaptic interfacial curvature [37]. At the pre-synapse terminals, numerous synaptic vesicles contained with neurotransmitters

move to attach the specific site of the pre-synaptic membrane known as the synaptic active zone or synaptic apposition and the neurotransmitters are released by exocytosis. The length of the active zone has been considered to reflect the effective area of synaptic contact and neurotransmitter releases [38]. After releases, the neurotransmitters are distributed in the synaptic cleft and then bind to their receptors at the postsynaptic membrane. The synaptic cleft is the gap between the pre-synaptic terminal and the post-synaptic membrane. It is contained with various molecules including neurotransmitters, various receptor molecules as well as the fibrillar elements which have a vital role in the regulation of synaptic cleft widths [39]. Since diffusion of neurotransmitters between neurons in the synaptic cleft can produce a membrane potential change in the postsynaptic membrane, thereby, the width of synaptic cleft is the great factor impact on the neurotransmission. Previous evidence has demonstrated that the narrow synaptic cleft is an indicating index for the enhancement of signal transmission [40]. The PSD is a resembling protein located under the postsynaptic membrane which is aligned with the synaptic apposition. It plays many critical roles in synapses including the regulation of adhesion, control of receptor clustering, and the regulation of receptor function [41]. An increase in thickness of PSD has wildly been demonstrated as enhanced synaptic strength [42]. In addition, the synaptic interfacial curvature is another morphological characteristic of synaptic plasticity. An increase in curvature interface of synapse enhances the proximity of receptor channels to the dendritic shaft or enlarges the contact area between neurotransmitter and the postsynaptic membrane, thereby improving the efficiency of transmission across the synaptic junction [43].

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Several studies have demonstrated that change in the synaptic function underlying learning and memory process relies on the alterations in the synaptic structure. The deterioration of learning and memory in Alzheimer's disease (AD) is thought to result from dysfunction and loss of synapses in the many brain regions

and concentrating in the hippocampus [44, 45]. Regarding an accumulative data, loss of the synapses became the best pathological correlation of cognitive impairment in AD [46-49]. However, previous pieces of evidence have provided that synaptic dysfunction arises at an early stage in neurodegenerative diseases such as AD followed by the synaptic loss in the late stage. Therefore, dysfunction and abnormality of synaptic signal transduction may be the key indicators in the early stage of neurodegenerative disease [50]. Interestingly, the synaptic structural parameters have wildly used to investigate the alteration of learning behavior. In 1981, Vrensen and colleagues had established that visual training led to an increased thickness of PSD and narrows the synaptic cleft at the supraoptic nucleus suggesting the structural plasticity of PSD and synaptic cleft are involved in learning modulation [51]. Recently, Hou and co-workers had revealed that the streptozotocin-administrated rats were shown the impairment of spatial memory and this deterioration was accompanied by an increase in the width of the synaptic cleft in the hippocampal CA1 region [40]. The previous study had also found that learning and memory deficit in toxic aluminum induction were related to synaptic cleft wider, less of a thickness of PSD, short in the length of the active zone, and smaller of the synaptic interfacial curvature in the hippocampus and frontal cortex [42]. These studies have provided evidence that the synaptic configuration changes in the key brain regions responsible for cognitive function are closely correlated with an alteration of learning and memory behaviors. The complication of the nervous system function relies on the appropriate working of the synaptic apparatus, and this function is controlled by various synaptic proteins which are existed in the subcellular compartments of the synapses. Loss of pre- and postsynaptic structural proteins such as synaptophysin (SYP) and postsynaptic density-95 (PSD-95), respectively, have well been documented in an impairment of synaptic plasticity [52, 53].

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2.3 Synaptophysin (SYP)

Synaptophysin (SYP) is a 38 kDa glycoprotein abundantly presented in the presynaptic membrane of neurotransmitter vesicles and in several neuroendocrine cells [54]. According to its mass approximately 10% of total vesicle protein, SYP is accepted to be the most predominant synaptic vesicle protein [54]. Together, SYP is exclusively localized to synaptic vesicles, it is widely used as a marker for presynaptic terminals [55]. This presynaptic protein serves a very vital role in the neurotransmission including movement of neurotransmitter (NT) vesicle, synaptic vesicle recycling and exocytosis of neurotransmitters [46, 56, 57]. The role of SYP on the NT secretory system is demonstrated in Figure 3. Previously, the changing of presynaptic vesicle proteins leading to amelioration of the secretory system has well been recognized [58]. Additionally, the expression of SYP is proposed to be implicated in synaptic plasticity and associated with the LTP induction [59, 60]. The previous study has as well demonstrated that loss of SYP is closely related to cognitive function decline in AD [61-63].

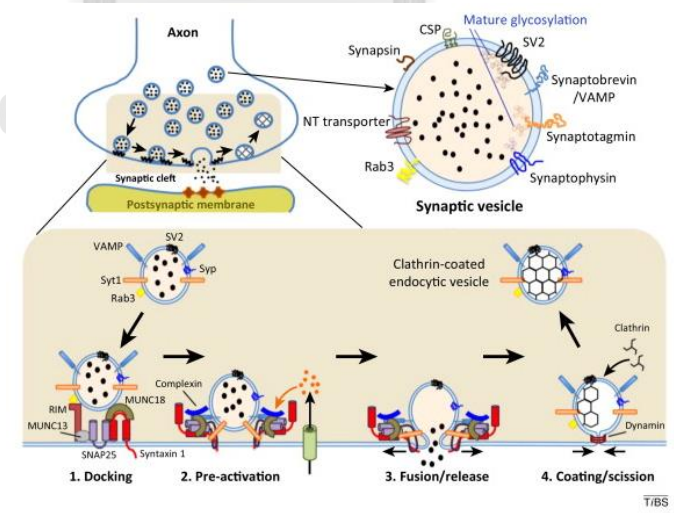

Figure 3 Schematic illustration of presynaptic molecules and dynamics. The localization of SYP is shown at the NT vesicle [64].

2.4 Postsynaptic density-95 (PSD-95)

Postsynaptic density-95 (PSD-95) is the most abundant scaffolding protein in the postsynaptic site. This protein is associated with N-methyl-D-aspartate (NMDA) glutamate receptor signaling and clustering of the receptor which is a critical role for synaptic plasticity [65]. Moreover, the PSD-95 is also a protein regulator for controlling the population of the α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor at the synapses [66], thereby, regulates the synaptic strength at the excitatory synapse [67-69]. Additionally, the large body of evidence has also demonstrated that there are numerous subcellular proteins binding to PSD-95 suggesting this protein displays a role in the protein molecular organization of the PSD [70-72] which is shown in Figure 4. The decreased level of PSD-95 has been indicated disrupting the function of the excitatory synapses [73].

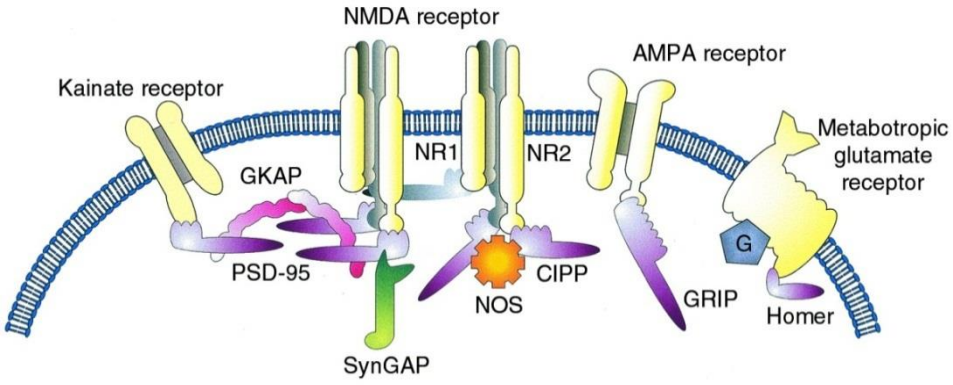

Figure 4 Interactions between glutamate receptors and PSD-95 protein [74].

2.5 Brain-derived neurotrophic factor (BDNF)

Several studies in the animal model have identified the necessary proteins for learning and memory and the predominant protein which is interested in this process is a brain-derived neurotrophic factor (BDNF). BDNF is the member of the

neurotrophin family which was first purified from the mammalian brain and classified as the member of the neurotrophin family [75]. The neurotrophins family has also consisted of neurotrophin-3 (NT3), neurotrophin-4/5, neurotrophin-6, and neurotrophin-7. Nevertheless, BDNF and its major receptor TrkB have the most abundant distributed in both the developing and adult mammalian brain with highest levels in the hippocampus and cerebral cortex [76, 77]. In the adult CNS, the action of neurotrophins have been widely studied and proposed to be involved in the LTP formation, a form of the synaptic plasticity associating learning and memory processes [23, 24].

BDNF is expressed in both presynapses and postsynapses. It can exert its effect as autocrine and paracrine mechanisms which rely on the site of cell surface receptors [77]. The mechanism underlying BDNF releasing has previously been proposed. Firstly, its releasing depends on the Ca^{2+} influx at the postsynaptic site following the activation of ionotropic glutamate receptors and voltage-gated Ca^{2+} channels [78]. Secondly, it can be released by Ca^{2+} influx at the presynaptic site. Thirdly, the releasing of Ca^{2+} from intracellular source can trigger the secretion of BDNF [66]. It has been revealed that the transport of BDNF (both mRNA and protein) into dendrites is regulated by the neuronal activity [79], and this process is suggested to be implicated in the modulation of synaptic transmission and synaptogenesis [80].

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The accumulative data have shown that various neuropathological conditions related to abnormalities in synaptic plasticity, such as neurodegenerative and neuropsychiatric disorders, are involved in the deficit in BDNF function [81, 82]. Furthermore, it has extremely been demonstrated that lack of BDNF in the key brain regions implicating learning and memory process was correlated to the impairment of cognitive functions [83]. The role of the BDNF is shown in Figure 5.

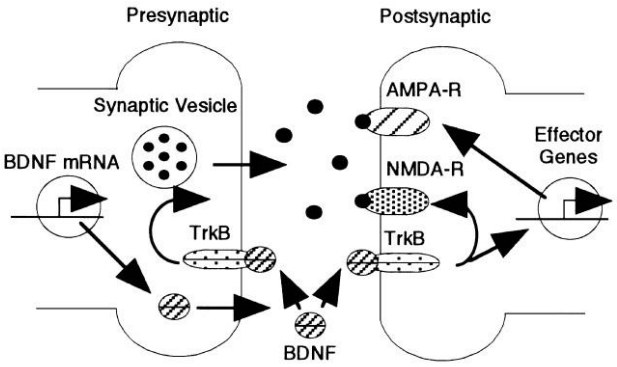

Figure 5 A drawing schematic model for the role of BDNF in learning and memory [84]

2.6 Oxidative stress

Oxidative stress is well known to be produced by the imbalance between the generations of free radicals including reactive oxygen species (ROS) and the oxidative defense known as antioxidants. During oxidative stress, the abundant of ROS, such as O^{2-}, OH^{-}, H_2O_2, NO and $ONOO^{-}$ are rapidly generated. In addition, oxidative metabolism of arachidonic acid is as well considered to be a significant source of ROS [85]. The accumulation of ROS can directly damage DNA, protein and lipid which finally results in cell damage and dysfunction which is shown in Figure 6. The brain is demonstrated to be particularly susceptible to ROS because it is the organ that utilizes oxygen more than other organs, rich in lipids with unsaturated fatty acids, and it is not particularly enriched with protective antioxidant enzymes of antioxidant compounds [86].

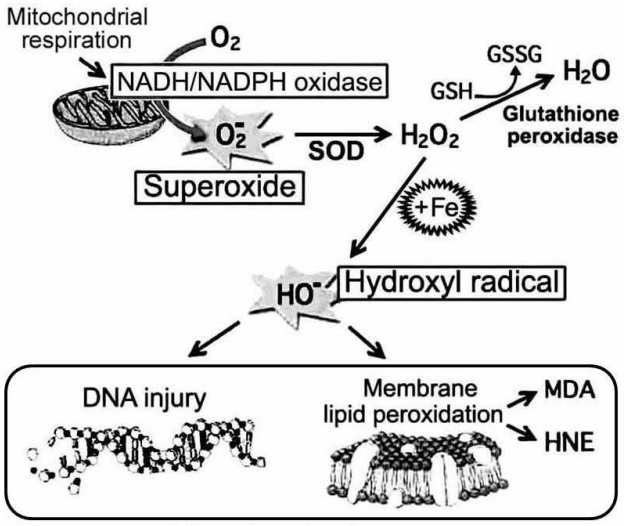

Figure 6 The diagram showing the oxidative stress induces cell damage [86]

Increased oxidative stress is considered to be implicated in the pathology of various neurodegenerative disorders including AD and Parkinson's disease (PD) [25]. Particularly, the association between increased oxidative stress and cognitive decline are widely established. In the animal experiment, the previous study has provided that oxidative stress induced by over fructose consumption in mice metabolic syndrome model showed a decrease in the synaptic active zone and PSD thickness in the hippocampus. These deteriorations were tightly correlated with the impairment of LTP-induced learning [23]. More and more evidence has also reported that ROS accumulation in the model of morphine induction could produce endoplasmic reticulum (ER) stress which resulted in dysregulation of synaptic balance including decreased synaptic numbers and reduced PSD-95 protein expression in the hippocampus suggesting the crucial role of ROS on the synaptic plasticity [87]. Furthermore, several previous studies have also proposed that the oxidative damage occurring at the protein levels was particular relevance to the progression of AD [88-90].

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Additionally, the previous study has demonstrated that increased oxidative stress contributes learning and memory deficit by which the effects are closely associated with a decreased BDNF protein and mRNA expression [26]. Although the relationship between oxidative stress and the BDNF expression is still not yet clearly elucidated, the probable mechanisms underlying oxidative stress reduce BDNF expression have previously been proposed. Some studies have hypothesized that increased oxidative stress can inhibit the DNA-binding activities of activator protein-1 and CREB resulting in decreased BDNF gene expression [90, 91]. Besides, increased oxidative stress can lead to the depletion of energy serving for NMDA channel function, thereby, the gene transcription of BDNF is decreased [92, 93].

Normally, there are many antioxidant enzymes acting as free radical scavenger which can mediate the level of ROS including superoxide dismutase (SOD), glutathione peroxidase (GPx), glutaredoxins, thioredoxins, and catalase. In addition to the enzymatic defense antioxidant mechanism, it has also been reported that the concentration of co-enzyme GSH in the cells and the activation of Nrf2 are strongly involved in the defense oxidative stress mechanism [94, 95].

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2.7 Glutathione (GSH)

Glutathione (GSH) is an important co-enzyme involved in the antioxidant defense mechanism which is presented in every cell types [94]. Besides, GSH has also multiple roles in various cell processing including cell division, the enzyme activity controlling, the regulation of transcription factor activity, and the modulation of ions to maintain the cell homeostasis [94]. In order to prevent an accumulation of ROS which can be generated by the physiological activities of the cell, in partially, GPX reduces various peroxides including H_2O_2 to be water (H_2O) by which the reaction requires two electrons form GSH [96].

GSH can be generally produced with two pathways; de novo synthesis and the recycling synthesis pathway [97]. The de novo synthesis of GSH is involved in maintaining the levels of GSH in the cells. In this pathway, the GSH is generated by the catalyzing of two cytosolic enzymes, **γ**-glutamylcysteine synthetase (**γ**GCS) and glutathione synthetase (GSS) with two steps. In the first step, the ligation of two amino acid, **γ**-glutamate, and cysteine, is formed to a **γ**-glutamylcysteine molecule catalyzed by the **γ**GCS enzyme. This step is feedback inhibited by GSH itself resulting in the rate-limiting step of GSH generation. In the second step, glycine is added to the C-terminal of **γ**-glutamylcysteine which is driven by GSS enzyme to form GSH. In another way, the resultant glutathione disulfide (GSSG), can be reduced back to GSH by glutathione reductase (GSR) via electron donation from the cofactor NADPH within the cells [98]. The roles and mechanism of GSH synthesis is demonstrated in Figure 7.

In the brain, the concentration of GSH is a range of 1-3 mM [98]. GSH is as well accepted to be a high capacity detoxification agent. It was demonstrated decreased GSH levels and elevated GSSG in the specific regions of the brain in various neurodegenerative disorders. As these existing evidence, it is suggested that both the decrement of GSH concentration and the increment of GSSG level allow increasing oxidative damage in cells and tissue in those disorders [99, 100].

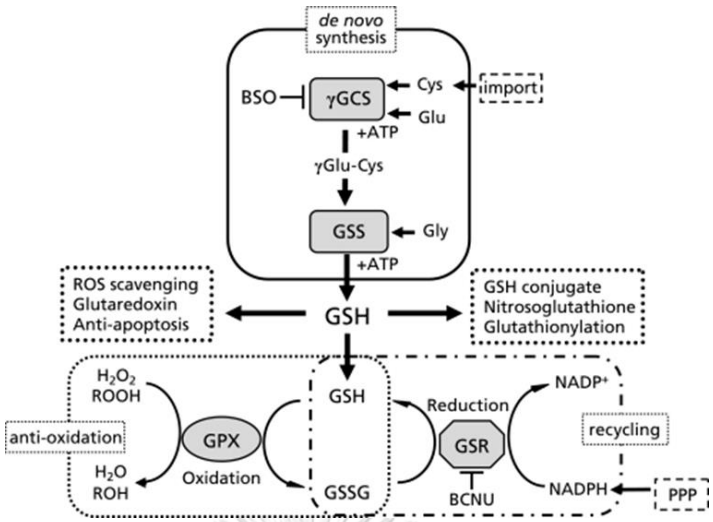

Figure 7 The schematic illustration of the roles of GSH in the antioxidant defense mechanism and their synthesis pathway [97]

2.8 Nuclear factor erythroid-2-related factor 2 (Nrf2)

Nuclear factor erythroid-2-related factor 2 (Nrf2) is a basic leucine zipper transcription factor which is a potent regulator of many antioxidant genes expression in the cells including γGCS, NAD(P)H quinone oxidoreductase 1 (NQO1), hemoxygenase (HO-1) [101]. In the normal condition, Nrf2 is suppressed by forming a complex with the stress sensor Kelchlike ECH associated protein 1 (KEAP1) in the cytoplasm. However, under the condition of oxidative stress, the KEAP1 is oxidized and modified by which it cannot bind to Nrf2, thereby resulting in Nrf2 release and translocate to the nucleus. Consequently, Nrf2 dimerizes with transcription factor Maf and binds to antioxidant response elements in the promoters of the genes that encode proteins involved in the antioxidant defense system [102]. The role s of Nrf2 is shown in Figure 8.

Although there are many Nrf2-dependent genes, it is reported that the dominant function of Nrf2 is involved in the activation of GSH-related gene

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transcription [94, 103]. Nrf2 promotes the transcription of the two subunits of GCS which is composed which a glutamate cysteine ligase (GCL)-modifier subunit (GCLM) and glutamate cysteine ligase (GCL)-catalytic subunit (GCLC). Besides the controlling of GCS expression, Nrf2 also regulates the abundance of cysteine within cells [104]. There is previous evidence suggesting that decreased Nrf2 level was demonstrated in AD patient despite the presence of oxidative stress [105]. However, other studies have revealed that an increase in the expression of the ARE-related genes was observed in patients with mild cognitive impairment and AD [106, 107]. Although the exact mechanism of Nrf2 in the various neuropathological conditions is still poorly understood, it was hinted that the fluctuation of Nrf2 might be manipulated by disease mechanisms [108]. However, in some studies, the overexpression of Nrf2 is suggested to become the potential therapeutic strategy for many neuropathological conditions such as Amyotrophic lateral sclerosis disease, PD and AD [105, 109].

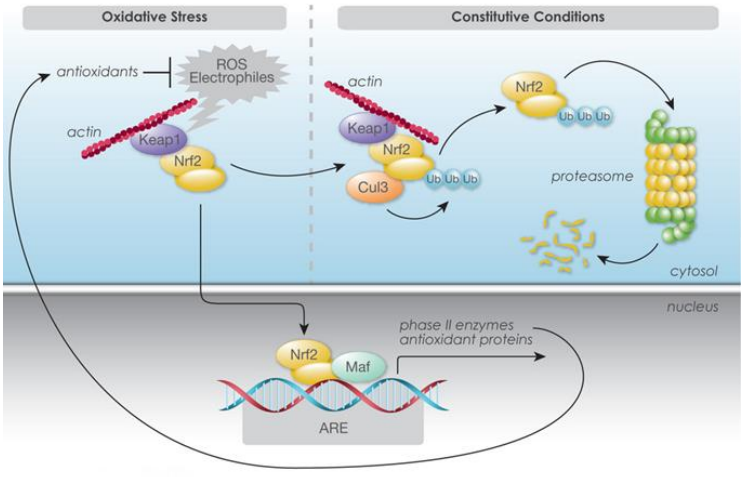

Figure 8 Schematic illustration of Keap1-Nrf2 antioxidant pathway [110]

2.9 Paracetamol (Acetaminophen: APAP)

Paracetamol (Acetaminophen: APAP) is accepted to be the most popular drug for treatment of pain and fever due to an inexpensive, high availability and minimal side-effects. Even though it is widely used for over 100 years ago, the mechanism of action is still not fully cleared [3]. However, several mechanisms underlying analgesic effects of this drug have been proposed as follow;

2.9.1 Mechanism of action

A central serotonergic mechanism

Previous evidence has proposed that the concentration of APAP in CSF was reflected in response for fever more than that in the blood circulation suggesting APAP has a potential effect in a central mechanism [111]. In the animal model, it has been suggested that the central mechanism underlying the analgesic effect of APAP are involved in 5-hydroxytryptamine type 3 (5-HT3) receptors [112]. This hypothesis was supported by the study in a human which confirmed by inhibiting with 5-HT3 receptor antagonists and that could block the analgesic effect of APAP [2]. Based on the results of this study, it is believed that APAP is able to exert its analgesic effect via descending inhibitory pain pathways [113].

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Prostaglandin H2 synthetase inhibition

It has previously suggested that the alternative pathway of APAP to produce the analgesic and antipyretic effects is involved in the inhibiting prostaglandins (PGH2) synthesis [114]. Commonly, there are two forms of enzymes that mainly produce the PGH2, constrictive PGHS-1 and the inducible PGHS-2, which known as COX-1 and COX-2, respectively [115]. Some evidence also suggested that there was

an alternative PGHS variant known as COX-3 presented in the CNS and it also is modulated by APAP [116].

Nitric oxide

Although the modulation of nitric oxide (NO) in nociception is still not fully understood, it was suggested that the nociceptive action may be concentration-dependent with high levels of NO [117]. The evidence obtained from the study in the animal has been proposed that APAP may exert its analgesic property at the peripheral levels through inactivation of spinal N-methyl-D-aspartate (NMDA) receptors. It was hypothesized that the inactivating NMDA receptor could reduce the synthesis of NO at the peripheral levels. Therefore, it is believed that APAP may produce anti-nociception which this process is involved in inhibition of NO mechanism [1].

Cannabinoid receptor activity

Previously, some evidence proposed that APAP can be metabolized and formed to be an active metabolite compound known as acidamide N-arachidonoyl-phenolamine or AM404. The metabolite AM404 has been considered to be implicated with cannabinoid (CBs) receptor activity to down the body temperature [118, 119]. However, the metabolite AM404 could not directly bind to CBs receptor, it was suggested to be a potent activator of the vanilloid subtype 1 receptor (TRPV1) [120], which is a ligand at CB1 receptors and an inhibitor of cellular anandamide uptake. These affect leads to an increased level of endogenous CBs and result in the inactivation of nociceptors [111].

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2.9.2 Metabolism of APAP

The major organ which implicated in the metabolism of APAP is liver and a lesser extend in kidney and intestine [121]. In the liver, after administrating with therapeutic dose, about 90% of APAP is metabolized by conjugation with glucuronic acid and sulfate to form water soluble molecules and excreted through urine [122]. About 5-10% of this drug can be metabolized by cytochrome P450 2E1 (CYP2E1) to form a reactive compound known as NAPQI which is considered to be as a potentially toxic to the cells [11]. After the generation, NAPQI is rapidly detoxified by interacting with GSH to form as non-toxic metabolite and then finally eliminated through the urine [9, 123]. In addition, it is also reported less than about 5% of APAP may be excreted via urine with unchanged form [124].

2.9.3 Effect of APAP treatment

Beneficial effects

Several studies have suggested that, at the therapeutic dose, APAP has protective effects in several pathological conditions. The experiment in cortical neuronal cell culture revealed that pretreatment with APAP at the concentration of 50 μM could promote neuronal viability which was accompanied by inhibiting cytokines and chemokines production induced by menadione exposure [5]. In the same year, this research group has as well provided that pretreatment with APAP at the concentration of 25-100 μM showed the protective effect against menadione-induced oxidative stress in the brain endothelial cells culture [6]. APAP was also reported for its anti-oxidative and anti-inflammatory activity in *in vitro* study. Pretreatment with APAP could protect hippocampal neuronal neurons from β-amyloid-induced oxidative stress by reducing the activation of nuclear factor-kappa B (NF-kB), a transcription factor which plays a key role in the regulation of many

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inflammatory genes [4]. Moreover, previous study has demonstrated that APAP could inhibit the status epilepticus (SE)-liked activity in primary hippocampal neurons and this effect was blocked by cannabinoid type 1 (CB1) receptor antagonist suggesting APAP exerts its effects via modulating CB1 receptors [125]. Furthermore, several previous studies have also proposed the beneficial effect of APAP treatment in animal experiments. In 2007, Ishida and collogues established that the mice received with APAP at the low dose (15.1 mg/kg bw) immediately after training showed increased spatial learning performance in MWM task and the effect was completely inverted by 5-HT(1/2) receptor antagonist. This study suggests that a nootropic property of APAP is implicated in 5-HT(1/2) receptor activity [17]. Recently, the study in cortical spreading depression (CSD) induction, a typical model of migraine headache, has provided the evidence that single injection of APAP at the therapeutic dose (200 mg/kg bw) could prevent an increased cerebral vessels alteration induced by CSD activation in rats [126]. As an accumulative data, APAP treatment, even at the therapeutic dose, has a potential effect to protect the cells or tissue from various stressors.

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Non-beneficial effect

It is well known that the hepatotoxicity is presented in the case of an excessive dose of APAP consumption. However, several studies have provided evidence that the APAP treatment as a non-toxic dose for liver had toxic in several organs or systems. Previously, there was data demonstrating that sub-toxic concentration of APAP treatment could reduce intracellular GSH in pulmonary macrophages and type II pneumocytes which might be a risk factor for asthma morbidity [7]. Likewise, a relationship between APAP consumption and an increase in the risk of asthma were also supported by the clinical study in both 20,000 children and adolescents in Spain. The results obtained from this study indicated that APAP

consumption was related to a significant increase in asthma symptoms and severity was observed in the frequent treatment with APAP [127]. The experiment in an animal has also confirmed the association of therapeutic APAP dose and the risk of chronic obstructive pulmonary disease (COPD) and asthma. This study showed that single and repeated treatment of APAP (15-60 mg/kg bw) produced an elevated neutrophil population, myeloperoxidase activity, as well as cytokine and chemokine levels in the mice airway. While the effect of APAP induced the inflammation were absent in the transient receptor potential ankyrin-1 channel (TRPA1)-deficient mice. Since TRPA1 is expressed by peptidergic primary sensory neuron and can be stimulated by NAPQI, this study suggested that airway tissue damaging effect of NAPQI may contribute to the risk of COPD and asthma associated with therapeutic APAP treatment [8].

Besides, the unwanted effect of this drug has also been proposed in the cardiovascular system. Several previous studies have demonstrated that chronic exposure to APAP, in the therapeutic doses, was associated with an increase in blood pressure. Clinical studies in both man and woman who frequently took APAP at a dosage of 500 mg/day demonstrated almost a 2-fold increase in the relative risk of incident hypertension than that observed in non-users [128-130]. Interestingly, chronic use of this drug can produce an increase in blood pressure was also supported by Sudano and colleges. Their results showed that treatment with APAP at a therapeutic dose (1 g/day) with standard cardiovascular therapy for 2 weeks in patients with coronary heart disease could induce an increase in both systolic and diastolic blood pressures [131].

During the last decade, there was evidence as well demonstrated the adverse effect of APAP consumption in the CNS. APAP is also widely used during pregnancy. The sibling-controlled study in Norwegian has shown that the children exposed

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chronically to prenatal APAP (more than 28 days) had poorer gross motor development and increase in the risk of adverse to psychomotor and behavioral outcomes such as attention deficit hyperactivity disorder (ADHD) [18]. In parallel, the recent animal experiment has shown that neonatal treatment with APAP resulted in cognitive deficiency and the alteration of anxiety-like behavior and drug response in adulthood. This study suggested that the mechanism underlying these effects of APAP treatment may be involved in the fluctuation of BDNF during brain development [19]. Moreover, several studies have demonstrated the adverse effect of this drug administration on the neuronal functions. It was revealed by a recent study that low dose APAP (10 and 50 mg/kg bw) consumption for 8 weeks could impair the profile of amino acids in several brain areas which may distribute the neurobehavioral disturbances [132]. The previous study has shown more evidence that a single high dose of APAP treatment (302.3 mg/kg bw) impaired MWM performance in mice. This effect has suggested the role of high-dose APAP in inhibiting the activity of COX-2 to impair memory [17]. However, the treatment of APAP in the concentration below to inhibit COX-2 activity could reduce synaptic-plasticity in hippocampal slice and this effect was reversed by 5-HT2/1 receptor antagonist, but not a 5-HT1 receptor antagonist. These results demonstrated that APAP modified the hippocampal synaptic plasticity via a presynaptic 5-HT2 receptor [133].

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Interestingly, it has previously been demonstrated that chronic APAP consumption could produce neuron damage in the hippocampus, the key brain region implicated in the processing of procedural learning and memory process. In this study, the rats received daily for 6 weeks with APAP at the dose of 100 mg/kg bw through drinking water caused neuronal damage in CA1 and CA3 region of the hippocampus and these effects were more severe in the combination with alcohol consumption [20]. In addition, Chantog and co-workers have reported the increase in

pro-inflammatory cytokines (TNF-α and IL-1α) in rat hippocampus after 30 days treatment with APAP [21]. Beside the effect on neuronal cells in the brain, a recent study has provided that chronic administration of APAP at the therapeutic dose (200 mg/kg bw for 30 days) caused the alteration of cerebral microvessels in cerebral cortex and these alterations were more severe when induced with CSD activation, but had no effect on the liver function [126].

Although the non-beneficial effects of this drug treatment on the CNS have continuously been reported, the mechanism underlying these effects is not yet clearly elucidated. In 2010, Posadas and co-workers have established the evidence that single intraperitoneal injection of APAP at doses below those required to induce hepatotoxicity (250 and 500 mg/kg bw) induced neuronal death in rat cerebral cortex. In this study they also found that APAP caused concentration-dependent neuronal apoptosis in *in vitro* experiment which the effect was associated with an increase in activity of neuronal CYP2E1 enzyme, cytochrome C releasing and caspase-3 expression suggesting the direct toxic of APAP in CNS [9]. In 2012, the same group of the researchers has again provided that the toxic of APAP to neurons is implicated in activating via the intrinsic pathway through a mechanism involving NF-kB which leads to an increase in IL-1β production [10]. Based on those results, they have suggested that the adverse effect of this drug treatment in CNS might be associated with the reactive metabolite of APAP which is converted by the CYP2E1 enzyme, NAPQI, in the brain.

As previously mentioned, NAPQI is accepted to be an extreme toxic metabolite of APAP [134, 135]. In the case of low dose levels, nearly all APAP is formed to be water soluble by conjugating with glucuronic acid and sulfate in the liver before eliminated via urine, while a small fraction of APAP is metabolized by the enzyme CYP2E1, which results in the formation of NAPQI [9, 136, 137]. After the

formation, NAPQI is always rapidly interacted with exist intracellular GSH to counter of poison before excreted through the urine [9, 123]. However, in the case of a large dose of APAP consumption, the accumulation of NAPQI in tissue might occur and it can also lead to the depletion of GSH which may result in oxidative stress [12, 13]. It was also reported that NAPQI itself can directly damage cells by binding with cellular proteins which can cause cell damage and dysfunction [11]. Together, the presented evidence has shown that enzyme CYP2E1 is distributed in several cell types in the brain including endothelial cells, astrocytes, pericytes, microglia, and neurons [9, 136, 137]. Since APAP can directly penetrate through the blood-brain barrier (BBB) [138], it can be distributed throughout the brain and also be metabolized by CYP2E1 to generate NAPQI in this organ. Based on this, abnormality of the cells in the brain and further alteration of neurobehaviors especially learning and memory abilities can be expected as a result of chronic or high concentration of APAP treatment.

In order to clarify this hypothesis, the present study aims to investigate the effects of APAP treatment, at the therapeutic dose (200 mg/kg bw), for the three different durations (0, 15 and 30 days) on the alteration of learning and memory in rats. The following alteration of learning and memory were monitored; the ultrastructural alteration of synaptic structural plasticity (the synaptic density, length of the active zone, width of the synaptic cleft and thickness of the PSD), the alteration of synaptic protein (SYP and PSD-95), the expression of BDNF in the frontal cortex and hippocampus. Furthermore, the levels of GSH concentration, the expression of Nrf2 and the levels of PCO content in both brain areas were as well monitored.

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CHAPTER III

MATERIALS AND METHODS

In order to investigate the effects of chronic APAP treatment on the alteration of learning and memory capacity, the animals in this study were treated as follows:

3.1 Animals

Male Wistar rats (250-300 grams) were purchased from National Animal Centre, Mahidol University, Thailand. The animals were housed in a stainless-steel bottom cage and maintained on normal rat food and tap water *ad libitum*. The temperature and humidity were maintained at 24 ± 1 ^{o}C with a 12-h dark/light cycle. All animals were received proper care and used in accordance with the Animals Ethical Committee of the Faculty of Medicine, Chulalongkorn University, Thailand.

3.2 Study design

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To investigate the effects of APAP treatment on the alteration of learning and memory in terms of different durations of treatment, this study was divided into three experiments.

Experiment I: This study aimed to investigate the effects of acute (0 day) APAP treatment on the alteration of learning and memory. The rats were sub-divided into two sub-groups (15 animals each) as follows: control and 200 mg/kg bw APAP treatment (APAP) groups. The rats in the APAP-treated groups were orally treated with APAP 1 hour before being evaluated with the NOR test, while distilled water at the same volume was fed to the rats in the control group. After finishing the NOR

test, all animals in this experiment were humanely killed and processed to perform the biochemical and ultrastructural analysis as described below.

Experiment II: This study aimed to investigate the effect of sub-chronic (15-day) treatment on the alteration of learning and memory. In this experiment, the rats were divided into two sub-groups (15 animals each) as follows: control and 200 mg/kg bw APAP treatment (APAP) groups. The rats in the APAP treatment groups were given 200 mg/kg bw APAP through oral gavage for 15 days, while the control rats were orally fed distilled water for the same period. Recognition memory was evaluated by the NOR test in all rats on the 8th day of treatment at 1 hour after the treatment. On the 9th day, all rats were processed for monitoring spatial learning and memory through the MWM test. One hour after individual daily treatments, the rats were trained to find the hidden platform for 6 days consecutively (from the 9th to 14th day of treatment). On the 15th day of treatment, their spatial memory was evaluated with the probe trial in all animals as described below. After finishing the MWM test, all animals in this experiment were humanely killed and processed to perform the biochemical and ultrastructural analysis as described below.

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Experiment III: This study aimed to investigate the effect of chronic (30-day) APAP treatment on alterations in learning and memory. The rats were sub-divided into two sub-groups (15 animals each) as follows: control and 200 mg/kg bw APAP treatment (APAP) groups. They were treated with similar protocols to those described in the second experiment except for the duration of the treatment. The rats were orally fed with APAP or distilled water for 30 days. All rats were processed for the NOR test on the 23rd day of the treatment. The rats all were trained from the 24th to 29th day of treatment, and their spatial memory was evaluated in the probe trial on the 30th treatment day. After finishing the MWM test, all animals in this experiment

were humanely killed and processed to perform the biochemical and ultrastructural analysis.

NB; The experimental design and schedule of this study are demonstrated in Figure 9 and 10, respectively.

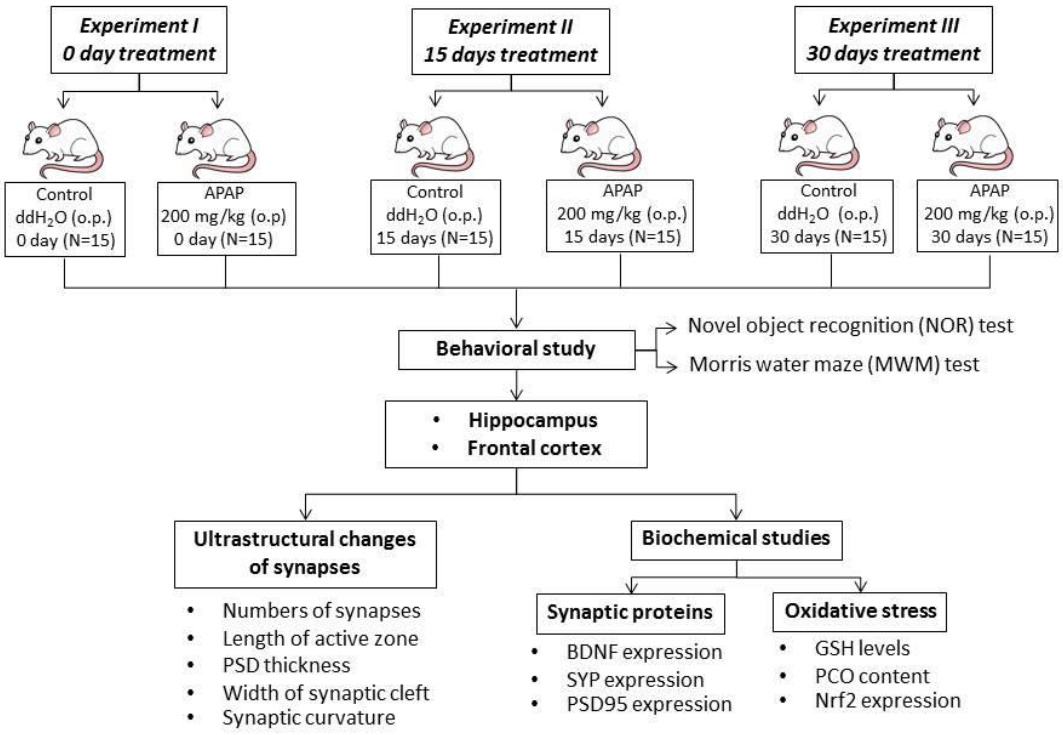
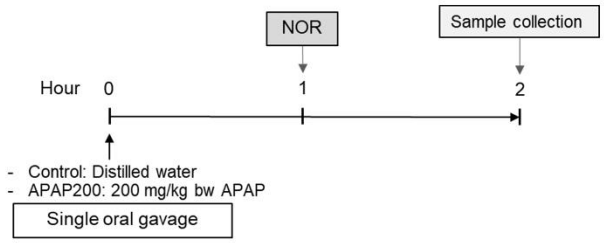
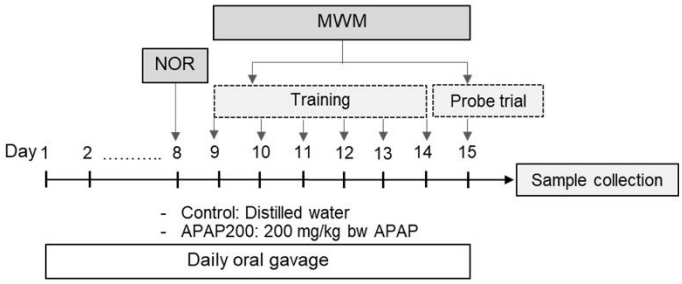


Figure 9 The diagram showing the experimental design

Experiment I:
Acute treatment



Experiment II:
Sub-chronic treatment



Experiment III:
Chronic treatment

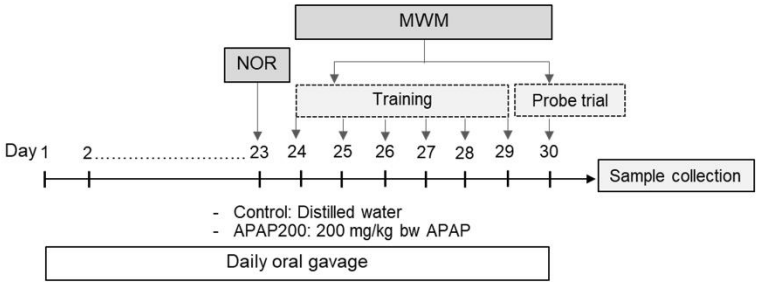


Figure 10 The diagram showing the experimental schedule

3.3 Behavioral study

3.3.1 Novel object recognition (NOR) test

The novel object recognition (NOR) test is the test for monitoring hippocampal and prefrontal cortical functions. This test has been established by Ennaceur and Delacuor since 1998. Based on the fact that if the animals can remember the familial object by their experience, they will spend the time to explore the new object more than a familial one [139].

In this study, the NOR assessment was done according to the modified procedure described by Morley et al. in 2001 [140]. The task was performed in an open black acrylic box with a 100 cm by 100 cm floor and a 50-cm high wall. All exploring behaviors of the animals were recorded by a video camera located above the arena throughout the testing. The test was performed at a time between 9:00 am to 4.00 pm and included three stages: habituation, training trial and testing trial. All rats were habituated to the arena in the absence of the object for 5 minutes and then returned to their cage for 1 hour. In the training trial, the ceramic cylinders (object A and B) were placed on opposite sides of the arena 20 cm from the arena wall. The rats were brought into the arena and left to freely explore the objects for 10 minutes. Next, the animals were returned to their home cage for 10 minutes. To perform the testing trial, one of the ceramic cylinders was substituted with a plastic toy (object C), which is considered to be a novel object prior to testing. The rats were placed in the area again to freely explore the objects for 10 minutes and then were returned back to their home cage. After finishing each trial for any rats, 70% ethanol was used to clean the arena and all objects to remove odors and olfactory cues.

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In this study, the exploring behaviors were defined according to the following reactions of the rats to the object: sniffing, rearing, or having their head directed toward the object within a 2-cm perimeter around the object. The schematic illustration of NOR test is demonstrated in Figure 11. The preference and recognition indexes were calculated with the following equations:

$$\text{The preference index (\%)} = \frac{\text{Total time spent for exploring object A}}{\text{Total time spent for exploring both objects during training trial}} \; x\; 100$$

$$\text{The recognition index (\%)} = \frac{\text{Total time spent for exploring object C}}{\text{Total time spent for exploring both objects during testing trial}} \; x\; 100$$

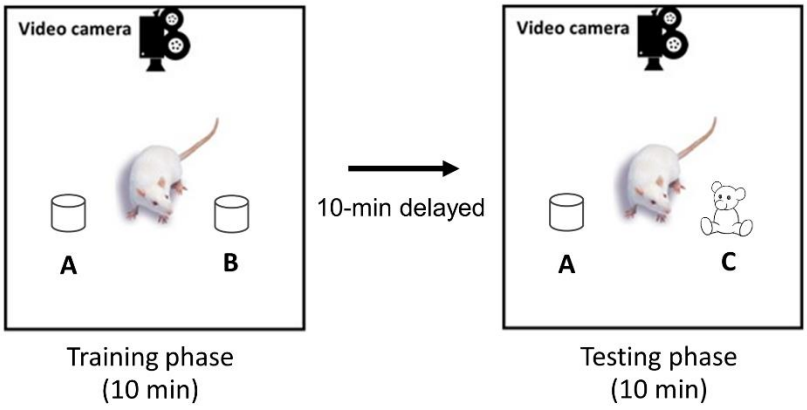

Figure 11 The schematic illustration of NOR test

3.3.2 Morris water maze (MWM) test

Morris water maze (MWM) test is a classical spatial learning and memory test for a rodent that depends on distal cues to navigate for reaching the target locations [141]. The spatial memory is predominantly associated with the hippocampus in rodents and humans [142]. MWM, therefore, is wildly used for access of hippocampal

dependent spatial navigation and reference memory and determine effects of various pharmacological treatments and brain damage [143, 144].

In this study, the MWM procedure was modified from the paradigm originally introduced by Morris in 1981 [141]. The test was performed between 12.00 am and 15.00 pm. A circular white pool with a 200-cm diameter and 60 cm deep was filled with water (23-24 ℃) to a height of 30 cm above the base. The pool was divided into four quadrants and labeled with different symbols that could be obviously seen by animals. A hidden platform with a 20-cm diameter was placed at the center of one quadrant throughout the training period. The platform had to be submerged 2 cm under the water surface. The digital video camera was located above the pool and was used to record the behavior during testing. In this study, the rats were trained to find the hidden platform for 6 days (1^{st} to 6^{th} day of the test), and three training trials were performed per day. In each trial, the rats were taken into the pool by placing a rat with its face toward the symbol labeled on the wall of the pool in the quadrant that did not have the platform (starting point). The rats were placed at a different starting point in each trial and were allowed to swim freely to find the hidden platform. If the rats could not find the platform within 90 seconds, they were guided to the hidden platform and placed on the platform for 15 seconds. The trial was terminated when the rat found the platform. At the end of each training day, all animals were removed from the pool, dried, and returned to their cages. The average time spent before finding the platform during the training trial was reported as the escape latency for the rat.

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After training for 6 days, spatial memory was evaluated. The hidden platform was removed, and the rats were allowed to freely swim for 90 seconds in the pool. The time spent within the target quadrant (the quadrant where the platform was

located) for each rat during the probe trial was recorded. The illustration of the MWM test apparatus is demonstrated in Figure 12.

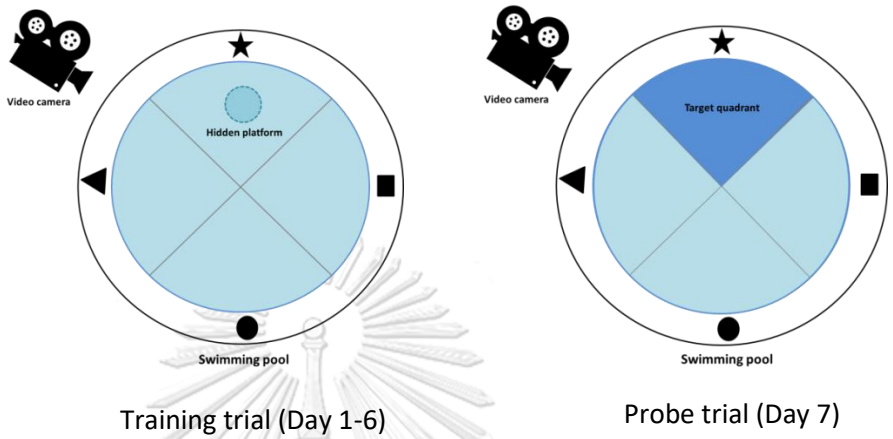

Figure 12 The schematic illustration of MWM test apparatus

3.4 Biochemical study

3.4.1 Samples collection

3.4.1.1 For GSH assay, PCO content measurement and western blot analysis

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Twenty-four hours after finishing of the behavioral test, all animals were deeply anesthetized with an excessive dose of sodium pentobarbital (60 mg/kg bw i.p.). The blood was collected by cardiac puncture method. After that, the transcardial perfusion with 0.1 M phosphate buffer saline (PBS) pH 7.4 at the volume of 250 mL was performed before decapitation and the brain was removed immediately. The frontal cortex and hippocampus were dissected rapidly on ice. The left side of the frontal cortex and hippocampus were collected for the GSH assay and PCO content measurement, while the right side of both the fresh brain tissues

were collected for western blot analysis. All of the collected brain tissues were frozen in liquid nitrogen and kept at -80 ℃ until assay.

3.4.1.2 For immunohistochemistry and transmission electron microscopy techniques

Twenty-four hours after finishing of the behavioral test all animal were perfused transcardially with 250 ml of 4% paraformaldehyde in 0.1M PBS pH 7.4 following the 0.1M PBS. The brain was removed out of the skull and immerged in the 4% paraformaldehyde at 4 ℃ for 48 hours before being further processed for embedding in the paraffin for immunohistochemical study. In addition, the right frontal cortex at the area of 3.70 mm anterior to bregma and the right hippocampus at the area of 3.60 mm posterior mm to bregma were cut into small cubes (1 mm^3) and then after submerged into 3% glutaraldehyde at 4 ℃ overnight before tissue processing for the transmission electron microscopic study.

3.4.1.3 Protein extraction

The frontal cortex and hippocampus were homogenized and sonicated in ice-cold RIPA lysis buffer (Cell Signaling Technology®, Massachusetts, USA), containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM b-glycerophosphate, 1 mM Na_3VO_4, 1 µg/ml leupeptin and protease/phosphatase inhibitor cocktail (Cell Signaling Technology®, Massachusetts, USA). The homogenates were then centrifuged 12,000 x g for 15 minutes at 4 ℃. The supernatants were collected and the protein concentration in the sample was determined by using the BCA protein assay kit (Thermo Scientific, Illinois, USA).

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3.4.2 Glutathione (GSH) assay

The GSH containing in the sample was detected according to the procedure as described by the manufacturer of the Cayman's GSH assay kit. Briefly, 50 ml of the sample or GSH standard was loaded to the 96 wells plate. The assay cocktail (volume 150 µL) containing 5, 5'-ditho-bis-2-nitrobenzoic acid, Ellman's reagent (DTNB) was added to each of the wells and incubated in the dark on an orbital shaker for 25 minutes. After that, the absorbance was measured at 405 nm. The difference in absorbance was calculated and the GSH concentrations were determined relative to the standard curve of the known concentrations of GSH. The levels of GSH were reported as nmol/mg protein.

3.4.3 Protein carbonyl oxidation (PCO) content measurement

The content of carbonyl oxidatively modified proteins was evaluated according to the method developed by Levine et al in 1990. The principal of their method is based on the reaction of the oxidized proteins with 2,4-dinitro-phenylhydrazine (DNPH) which will result in the forming of hydrazones [145]. Briefly, 62.5 µL of the sample was incubated with 250 µL of 10 mM DNPH solution in the dark for 60 minutes at room temperature. After that, the proteins were precipitated by the incubation of 300 µL of 20% (v/v) trichloroacetic acid (TCA) at 4 °C for 10 minutes followed by centrifuge at 10,000 x g for 15 minutes. Then the protein pellet was washed three times with ethanol and ethyl acetate (1:1 v/v) and re-suspended in 300 µL of 6 M guanidine at 60 °C for 1 hour. The absorbance was read at 370 nm. The quantification of PCO content was calculated using the following equation:

$$\text{Protein carbonyl oxidation (nmol/mg protein)} = \frac{\Delta 375 \text{ x } 45.45 \text{ (nmol/mL)}}{\text{Total protein concentration (mg/mL)}}$$

3.4.4 Western blot analysis

In this study, the expression of BDNF, SYP and PSD-95 were detected. Equal amounts of protein samples (10-40 μg) were mixed with loading buffer and boiled at 95 ℃ for 5 minutes. The protein was electrically separated in 7.5-15% SDS polyacrylamide gel and electrically transferred onto the nitrocellulose membranes. The blots were blocked non-specific binding of proteins with 5% non-fat dry milk or 5% bovine serum albumin (BSA) in Tris-buffered saline containing with 0.1% Tween-20 (TBST) for 1 hour at room temperature. The blots were incubated with primary rabbit anti-BDNF antibody (1:500 dilution; Abcam, Cambridge, MA), mouse anti-SYP antibody (1:1,000 dilution; Merck Millipore, Massachusetts, USA), rabbit anti-PSD-95 antibody (1:2,000 dilution; Cell Signaling Technology®, Massachusetts, USA) or mouse anti-β-actin antibody (1:3,000 dilution; Sigma, St. Louis, Missouri, USA) at 4 ℃ overnight with a gentle agitation for the detection of BDNF, SYP and PSD95, respectively. After washing the membranes 3 times for 10 minutes each, secondary anti-mouse or rabbit antibody conjugated with horseradish peroxidase (HRP) (Sigma, St. Louis, Missouri, USA) were incubated to the membrane for 1 hour at room temperature. After washing each membrane, immunoreactive bands were visualized using a chemiluminescence system (Amersham ECLTM Prime Western Blotting Detection Reagent, GE Healthcare Life Sciences, Buckinghamshire, UK) and exposed onto film. The immunoreactive band densities were analyzed by using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). The results were represented as the ratios of the densities all of the target proteins to the internal loading control protein, β-actin.

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3.4.5 Immunohistochemistry

The paraformaldehyde-fixed brains (5 per group) were dehydrated in ethanol series, processed and embedded in paraffin wax. Each paraffin-embed block of the brain was coronally sectioned at the thickness of 5 μm. The sections were collected in a series of 1 in 8 (2 sections were collected from each animal) and placed on Superfrost plus slides (Thermoscientific, Portsmouth, New Hampshire, USA). All sections were deparaffinized and incubated with antigen retrieval solution (citrate buffer pH 6.0, Dako, Glostrup, Denmark), 3% Hydrogen peroxide, and 3% normal horse serum (PAN Biotech GmbH, Aidenbach, Germany) in PBS. The sections were incubated with primary rabbit anti-BDNF antibody (1:2,000; Abcam, Cambridge, UK) at 37 °C for 37 minutes. The BDNF immunoreactivity was detected using an ultraView Universal DAB Detection Kit (Ventana Medical Systems, Inc., Arizona, USA). The entire process was conducted with an automatic slide staining machine (Benchmark XT, Ventana Medical Systems, Inc., USA). All slides were counterstained with hematoxylin, dehydrated in ethanol series, mounted, and cover-slipped with a mounting media. All sections were scanned using a slide scanner (Aperio ScanScope, Aperio, Vista, California, USA). The immunoreactivity of BDNF in the hippocampus (approximately -3.14 to 3.30 from bregma) and mPFC (approximately 2.70 to 3.20 from bregma) were monitored by using the positive pixel counting algorithms (v9.1, Aperio, Vista, California, USA). The collected brain areas which for evaluating the expression of BDNF protein are shown in Figure 13. During analysis, investigators were blinded as to the identity of all samples.

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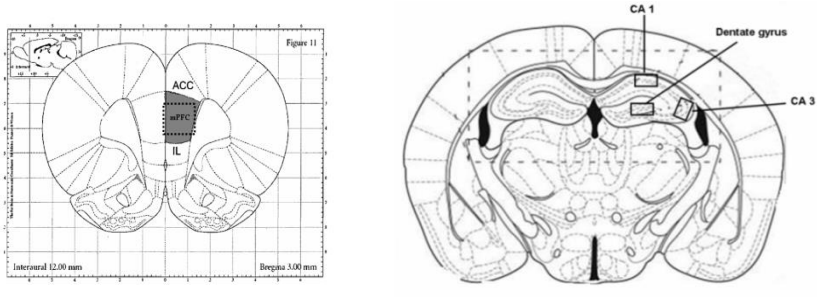

mPFC Hippocampus

Figure 13 The schematic illustration of the areas of the mPFC and hippocampus for investigating the BDNF expression in immunohistochemistry.

3.4.6 Immunofluorescence assay

To determine the expression and localization of Nrf2 protein in the mPFC and hippocampus, the paraffin-embedded tissue sections were prepared as described previously. All the sections were deparaffinized and rehydrated and then further incubated with antigen retrieval solution (citrate buffer pH 6.0, Dako, Glostrup, Denmark) and 3% normal horse serum (PAN Biotech GmbH, Aidenbach, Germany) in PBS. The sections were treated with primary rabbit anti-Nrf2 antibody (1:200 dilution; Abcam, Cambridge, UK) at 4 ℃ overnight. After washing, the sections were further incubated with Alexa Fluor® 488 anti-rabbit IgG secondary antibody for 2 hours. Then, the sections were incubated with DAPI for nuclear counterstaining. Finally, the sections were coverslipped with a cover glass and further examined under a fluorescence microscope (Olympus, Tokyo, Japan).

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3.5 Transmission electron microscopic study

All 1 mm^3 frontal cortex and hippocampus cubes were post-fixed with 1% osmium tetroxide and dehydrated through a graded series of ethanol. The tissues

were passed through propylene oxide and embedded in Epon (Epon 812; Electron Microscopy Sciences, Ft. Washington, USA). The area of layer II/III of the pyramidal neurons of mPFC and stratum radiatum (SR.) of CA1 were selected to investigate the synaptic ultrastructural changes in the frontal cortex and hippocampus, respectively. The ultra-thin sections (70-90 nm thick) were cut by the ultramicrotome, placed on a copper grid and stained with uranyl acetate and lead citrate. The stained sections were examined under the transmission electron microscope (JEM 1210; JEOL, Tokyo, Japan).

The ultrastructural changes of synapses related with learning and memory process including the number of synapses, the length of the active zone, the width of the synaptic cleft, the thickness of the PSD, and the curvature of the synapses were monitored in this study. In order to determine the synaptic ultrastructural changes, the typical Gray I synapse, the excitatory neurotransmitter glutamate which is predominantly implicated in synaptic plasticity, was identified and selected. This type of the synapse is characterized by the presence of a typical asymmetric interface (i.e. the thickness of the postsynaptic membrane is much thicker than the presynaptic membrane), the appearing of postsynaptic density in the postsynaptic membrane, and the showing round and clear synaptic vesicles [146]. The illustration of the typical Gray I synapse is shown in Figure 14. In order to monitor the numbers of the synapses, two sections were randomly selected from each animal (5 animals in total per group) and ten montage electron micrographs (per animal) were taken at the magnification of 8,000X. The density of synapses was represented as the average number of synapses per area.

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In order to quantitative evaluate the synaptic interfacial parameters including length of the active zone, the width of the synaptic cleft, the thickness of the PSD, and the synaptic curvature, thirty synapses from each animal were randomly selected, and electron micrograph of each synapse was taken at a magnification of

50,000X. Then four synaptic interfacial parameters were investigated as the following criteria;

1. The length of the active zone was monitored by measuring the length between two ends of the presynaptic membrane where the synaptic vesicle docking presented [146].

2. The width of the synaptic cleft was analyzed by averaging the widest and narrowed portions of the synapses [147].

3. The thickness of the PSD was determined by averaging the three points measuring the PSD thickness. In most case of PSD thickness consists of a highly electron dense portion on the postsynaptic membrane and a moderately electron dense, which usually extends somewhat further into the dendritic cytoplasm [148].

4. The synaptic interfacial curvature was evaluated using the formula: R = a/b, where ‘R’ is synaptic curvature, ‘b’ is the line joining the two end of the postsynaptic thickening and ‘a’ is the perpendicular distance from the postsynaptic membrane to ‘b’ [149].

The measuring of synaptic interfacial curvature is demonstrated in Figure 15. All data of the synaptic interfacial structural parameters were expressed as the average of all synapses in each photo taken at a magnification of 50,000X.

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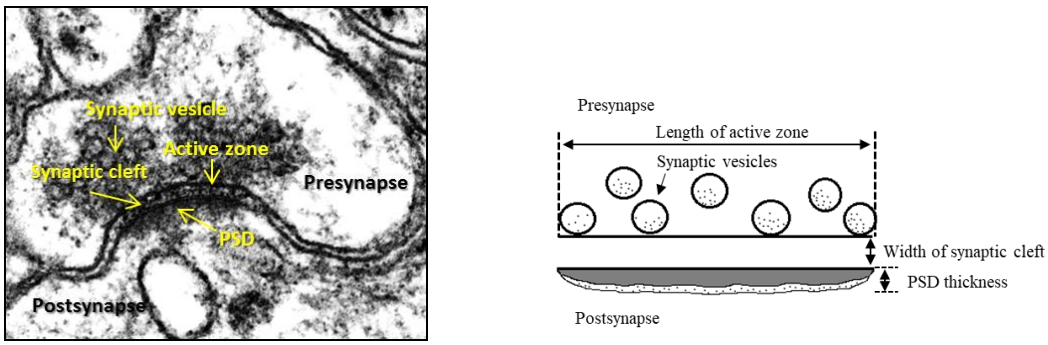

A. B.

Figure 14 The illustration of the typical Gray I synapse. The electron micrograph of the synaptic interfacial structure (A) and the schematic drawing of the synaptic interfacial structure (B).

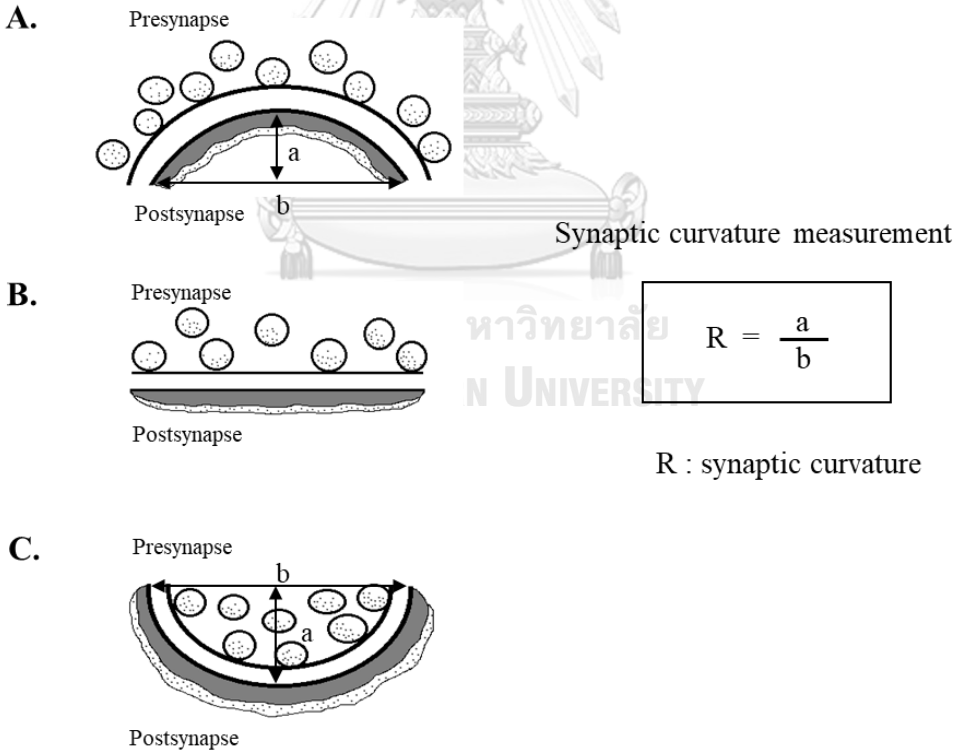

Figure 15 Scheme illustrating the synaptic curvature measurement for the convex synapse (A), flat synapse (B) and concave synapse (C).

3.6 Data analysis

The results were presented as means ± the standard error of mean (S.E.M.). Statistical analyses were performed using the unpaired Student's t-test for two groups comparison and the one-way ANOVA followed by Bonferroni's test for multiple comparison tests (SPSS ver. 22). The probability values (*P*) values of less than 0.05 was considered statistically significant.

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CHAPTER IV

RESULTS

In this study, APAP at a dose of 200 mg/kg bw was used as a drug treatment in the animals. The results revealed that neither acute nor chronic APAP treatment affected the rats' body weights (results not shown). Our results also demonstrated that 30 days of APAP oral administration did not affect the three main enzymes associated with the liver function (alanine aminotransferase; AST, aspartate aminotransferase; ALT and alkaline phosphatase; ALP) and the morphology of the liver showed no significant differences between the APAP-treated animals as compared with those in the control group suggesting chronic treatment with APAP at the dose of 200 mg/kg bw had no any effect on the liver function (data not shown).

The results obtained from the present study are divided into two parts. The first part is the results obtained from the behavioral study, while the second part is the results obtained from the biochemical and histological structural studies as follows:

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5.1 Behavioral study

5.1.1 Effect of APAP treatment on NOR test

Effect of 0-day APAP treatment on NOR test

The results obtained from NOR test in the experiment with acute treatment demonstrated that APAP treatment had no effect on the preference and recognition index. The time spent for exploring the objects during the training phase was not different between the rats treated with APAP and control group (Figure 16A).

In addition, there was no significant difference in the time spent for exploring the novel object during the testing phase in acute APAP treated group when compared to the control group (Figure 16B).

Effect of 8-day APAP treatment on NOR test

Using the NOR test, the results obtained from the experiment with 8-day treatment demonstrated no any difference in the preference index between the APAP-treated rats and control group. The exploring time with the object in the training phase of the rats in APAP-treated group was not significantly different compared with those in the control group (Figure 17A). However, a reduction of the recognition index was observed in the 8-day APAP-treated rats as compared to those in a control group. The result revealed that the time spent to explore the novel object during the testing phase of rat treated with APAP was significantly lower than that observed in the control group ($P<0.05$, Figure 17B).

Effect of 23-day APAP treatment on NOR test

In the experiment with 23-day treatment, the results obtained from NOR test demonstrated that chronic treatment with APAP had no effect on the preference index. The time spent to explore the objects during the training phase in the rats received chronically with APAP was not significantly different as compared with those in the control group (Figure 18A). However, the observation during the testing phase demonstrated a reduction of the recognition index in the rats with chronic APAP treatment. The results showed that the time spent for exploring the novel object of the rats treated chronically with APAP was significantly decreased when compared to the rats in the control group ($P<0.05$, Figure 18B).

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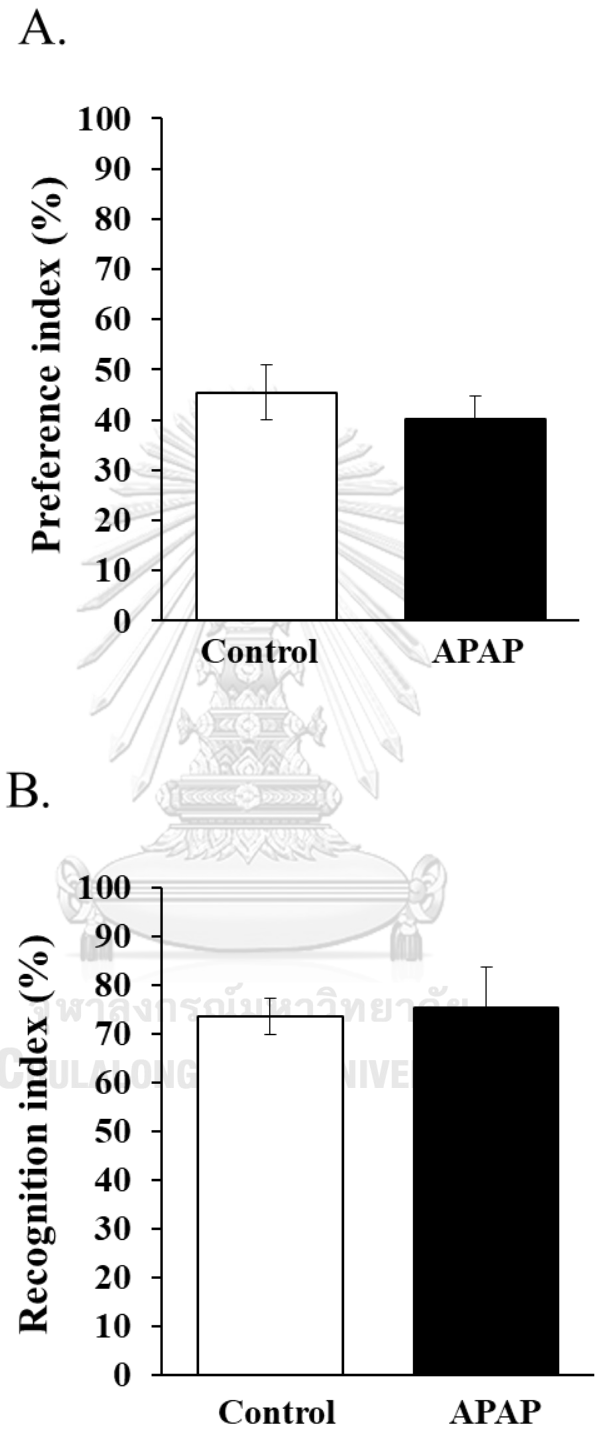

Figure 16 Effect of 0-day APAP treatment on the alteration of NOR performance in rats. The preference index (A) and recognition index (B) of control and APAP-treated rats. The values are expressed as mean ± SEM.

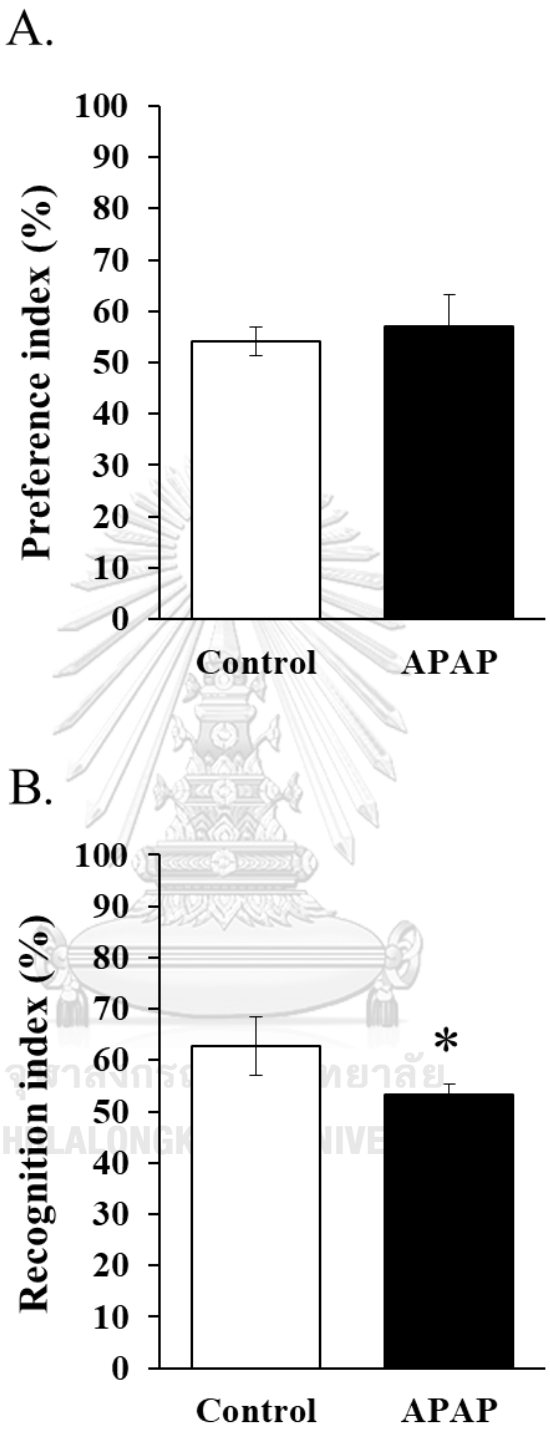

Figure 17 Effect of 8-day APAP treatment on the alteration of NOR performance in rats. The preference index (A) and recognition index (B) of control and APAP-treated rats. The values are expressed as mean ± SEM. *P<0.05 compared to the control group.

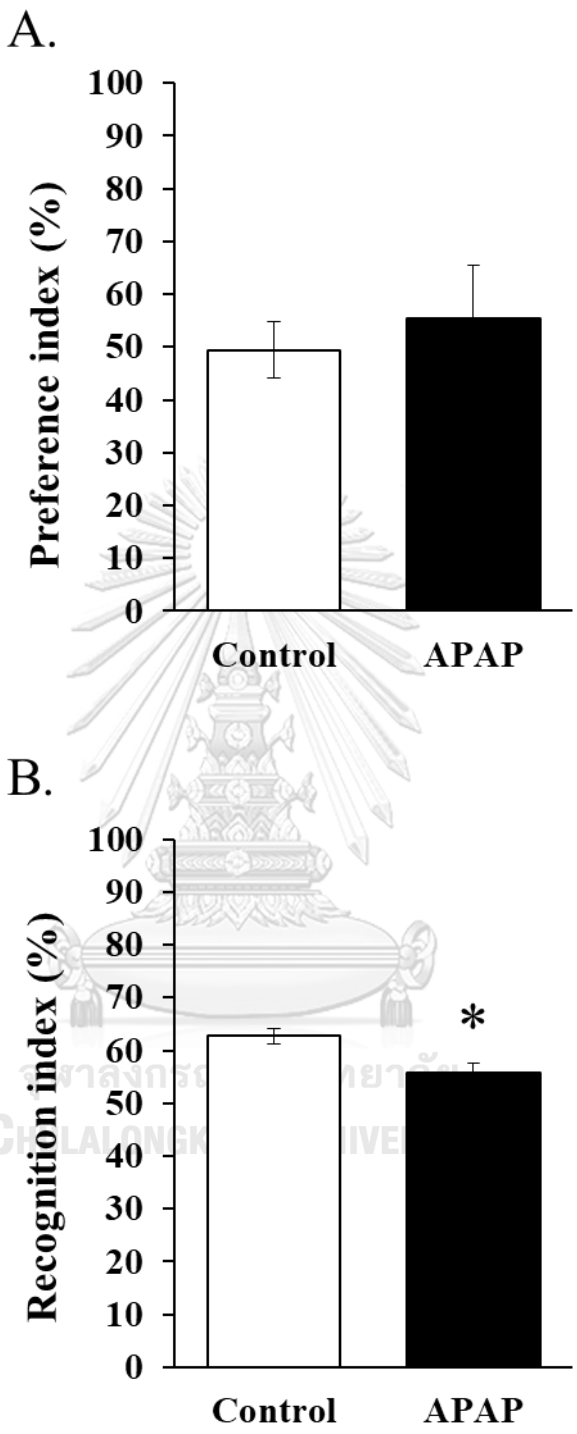

Figure 18 Effect of 23-day APAP treatment on the alteration of NOR performance in rats. The preference index (A) and recognition index (B) of control and APAP-treated rats. The values are expressed as mean ± SEM. *P<0.05 compared to the control group.

5.1.2 Effect of APAP treatment on MWM test

In order to evaluate the MWM performance, animals needed to be trained consecutively for 6 days, therefore, the MWM performance was not monitored in the rats with acute APAP treatment.

Effect of 15-day APAP treatment on the MWM test

The results demonstrated a significant difference in the escape latency between the experimental groups. The escape latency in 15-day APAP treated rats was significantly higher than that observed in the control on the 2nd and 5th day of the training session (P<0.01 and P<0.05, respectively, Figure 19A). Furthermore, the results obtained from the probe trial revealed that time spent in the target quadrant of the 15-day APAP-treated group was significantly lower than that in the control group (P<0.05, Figure 19B).

Effect of 30-day APAP treatment on the MWM test

In the 30-day treatment, the results obtained from the MWM test showed that chronic treatment with APAP could reduce the spatial learning and memory performance in the rats. The escapes latency obtained from the rats received chronically with APAP were significantly higher than those in the control group on the 1st and 3rd day of the training session (P<0.01 and P<0.05, respectively, Figure 20A). In addition, the results also demonstrated that the rats in the chronic APAP treatment showed a decrease in spatial memory. It was found that the time spent in the target quadrant during the probe trial obtained from the 30-day APAP-treated group was significantly lower than that in the control group (P<0.01, Figure 20B).

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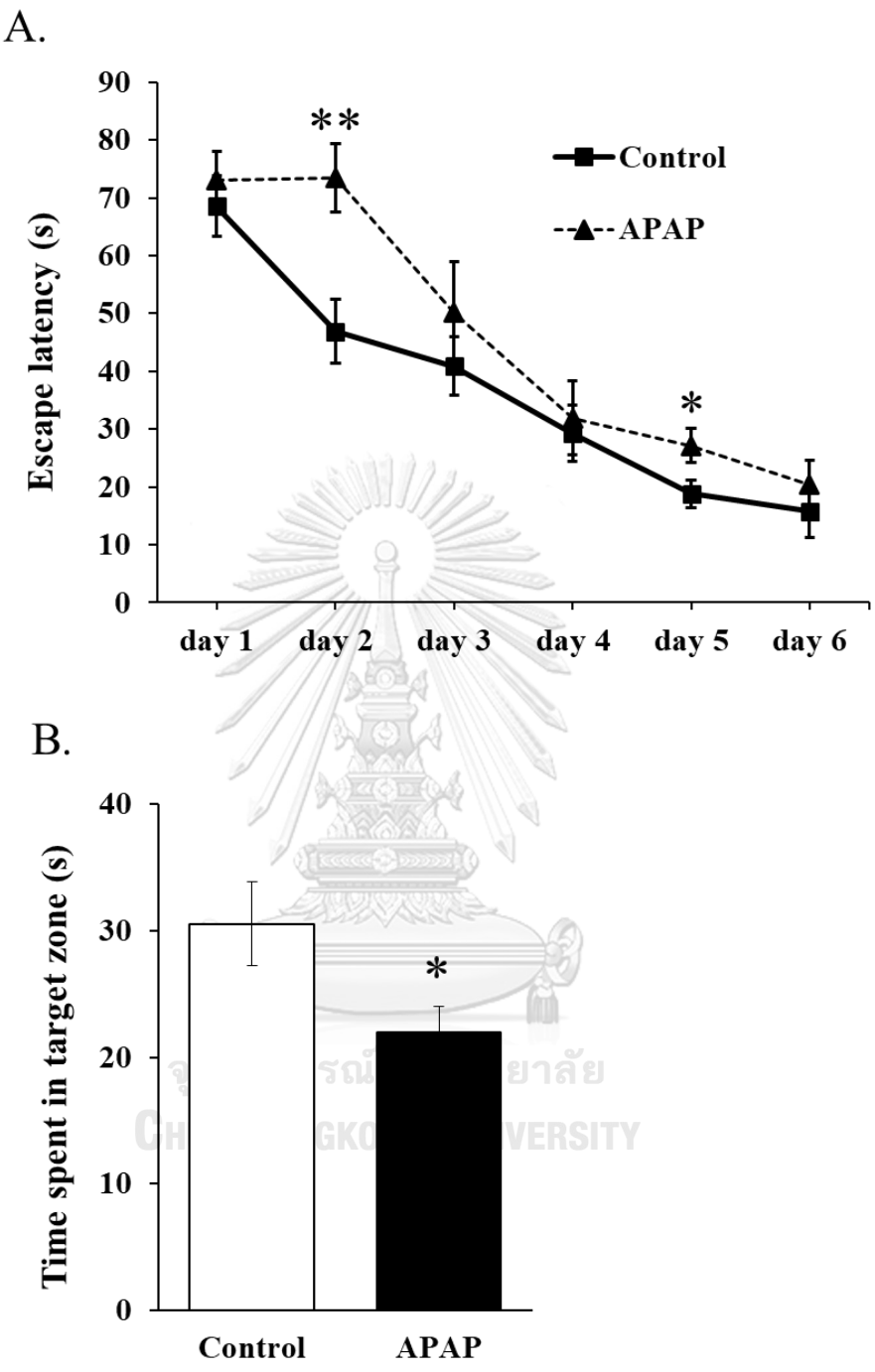

Figure 19 Effect of 15-day APAP treatment on the alteration of MWM performance in rats. The escape latency during the training trial (A) and time spent in the target quadrant during probe trial (B) of control and APAP-treated rats. The values are expressed as mean ± SEM. *P<0.05 compared to the control group.

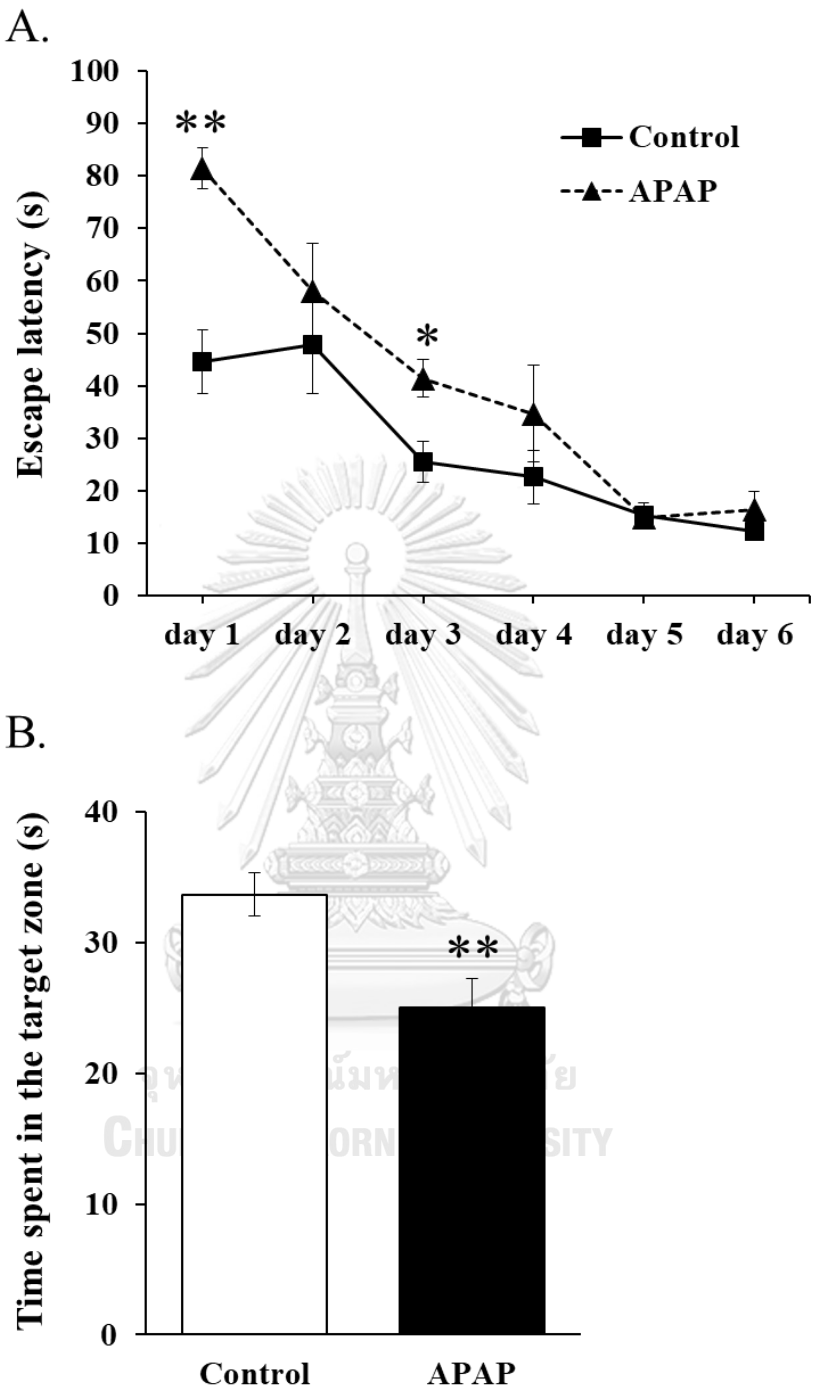

Figure 20 Effect of 30-day APAP treatment on the alteration of MWM performance in rats. The escape latency during the training trial (A) and time spent in the target quadrant during probe trial (B) of control and APAP-treated rats. The values are expressed as mean ± SEM. *P<0.05 and **P<0.01 compared to the control group

5.2 Structural and biochemical studies

5.2.1 Effect of APAP treatment on the alteration of synaptic ultrastructure using transmission electron microscope study

Synaptic plasticity is widely accepted to be closely associated with cognitive function. Therefore, in this study, the alterations of synaptic ultrastructure related with synaptic transmission were monitored including the numbers of synapses, length of the active zone, width of the synaptic cleft, thickness of the PSD and the synaptic interfacial curvature in the key brain regions responsible for learning and memory process were determined in this study.

Effect of APAP treatment on the number of synapses

Using the TEM study, the representative series of electron micrographs of the synaptic density in the mPFC (layer II/III pyramidal neurons) and hippocampal CA1 (SR. layer) are shown in Figure 21 and 22, respectively.

In the present study, the quantitative analysis of the synaptic density demonstrated that neither acute nor sub-chronic treatment (0 and 15 days) with APAP could change the synaptic density in the mPFC and hippocampus. Our results revealed that there was no significant difference in the numbers of synapses in both brain areas of the rats treated with APAP as compared to the control group (Figure 23). However, a study in the chronic treatment (30 days) demonstrated that the density of synapses in the mPFC and hippocampus of the rats treated chronically with APAP were significantly lower than those in the control group ($P<0.05$, Figure 23).

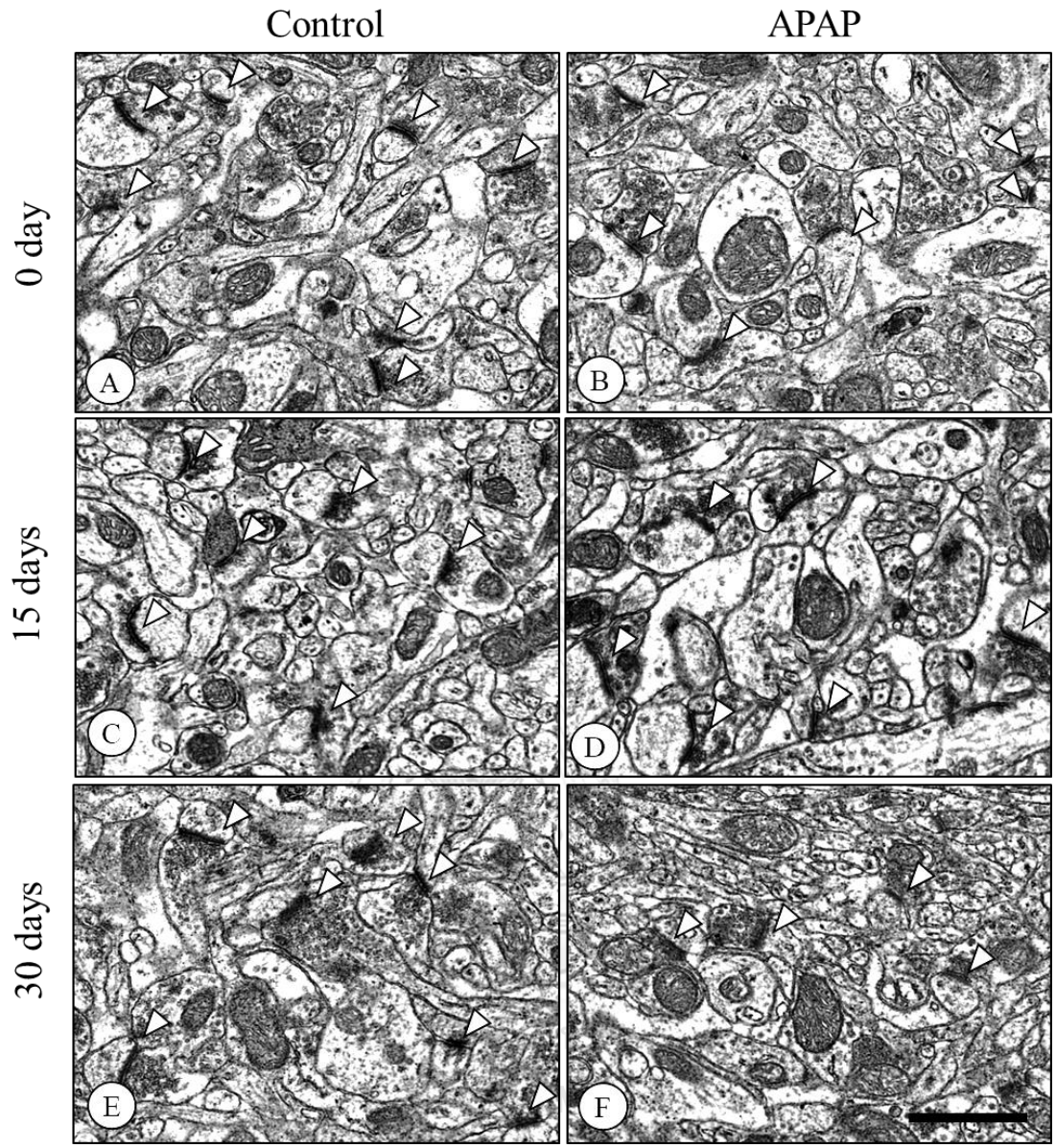

Figure 21 Effect of APAP treatment at the three different durations (0, 15 and 30 days) on the synaptic density in the mPFC. TEM micrographs of the mPFC sections obtained from the control groups (A, C and E) and APAP-treated groups (B, D and F). The synaptic ultrastructure in the electron micrographs are indicated by the white arrow heads; scale bar = 1 μm.

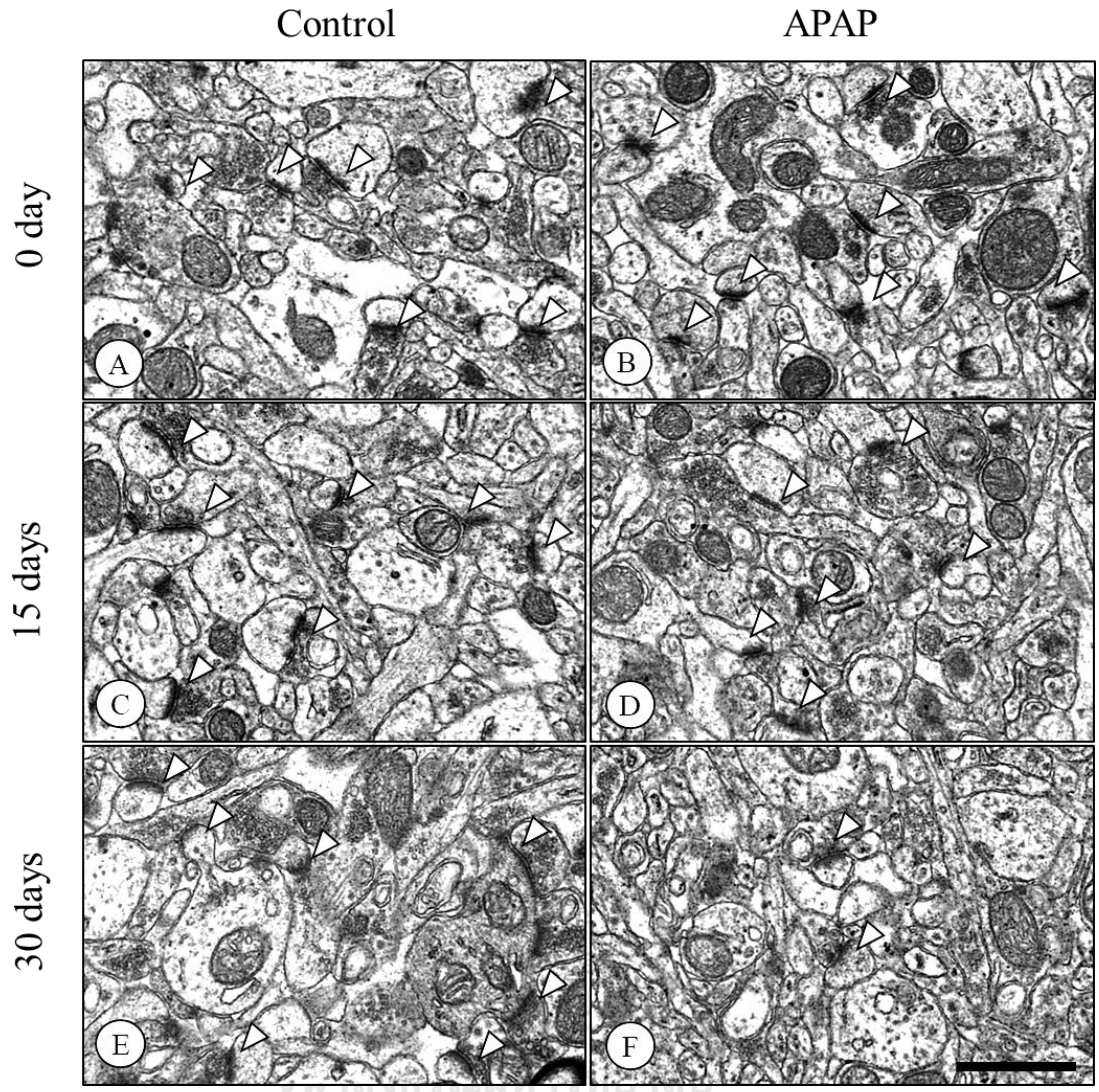

Figure 22 Effect of APAP treatment at the three different durations (0, 15 and 30 days) on the synaptic density in the hippocampal CA1 region. TEM micrographs of the hippocampal sections obtained from the control groups (A, C and E) and APAP-treated groups (B, D and F). The synaptic ultrastructure in the electron micrographs are indicated by the white arrow heads; scale bar = 1 μm.

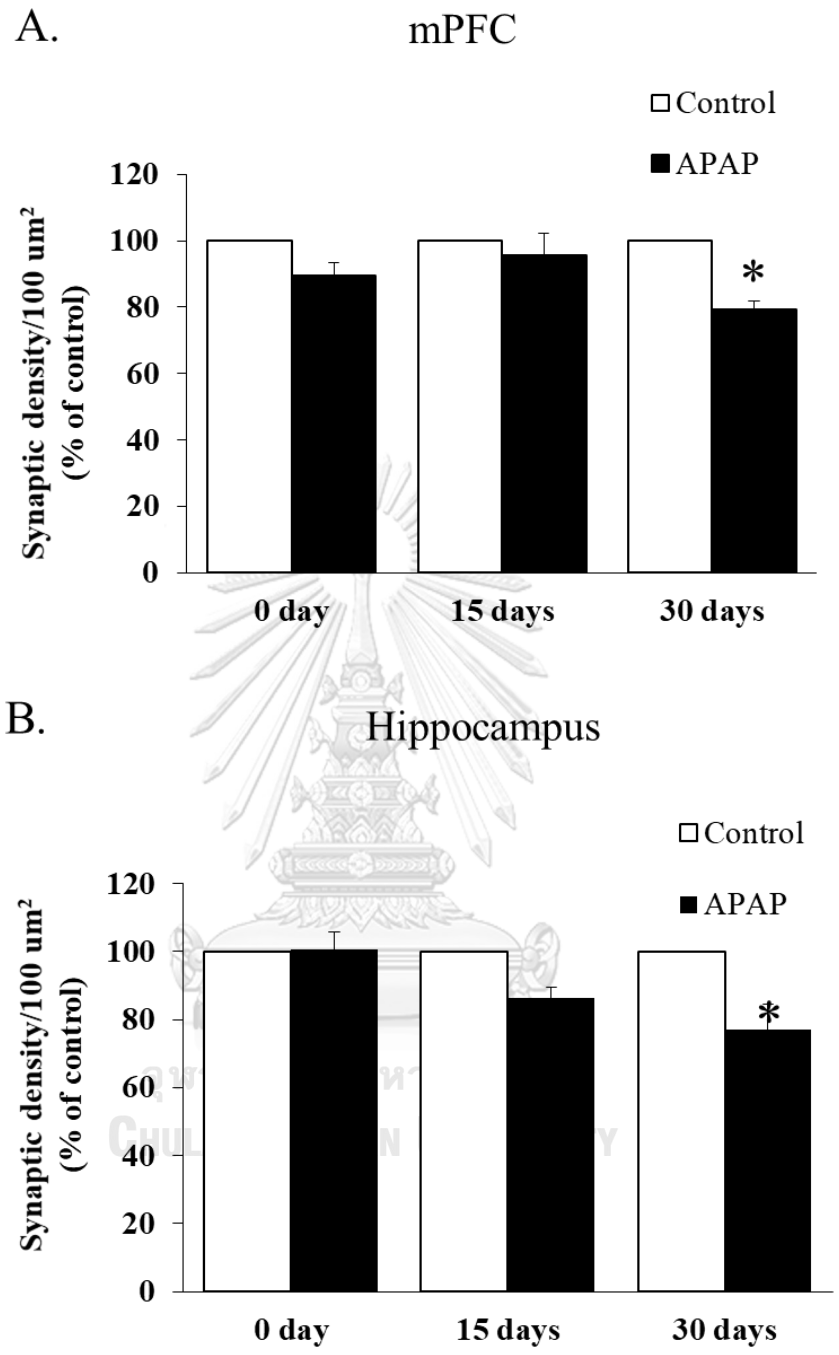

Figure 23 The effect of APAP treatment at the three different durations (0, 15 and 30 days) on the numbers of synapses. The numbers of synapses in the mPFC (A) and CA1 region of the hippocampus (B) obtained from the control and APAP-treated rats. The values are expressed as the mean ± SEM. *P<0.05 compared to the control group.

Effect of APAP treatment on the synaptic ultrastructure

The representative series of electron micrographs of all the synaptic ultrastructural parameters in the mPFC and hippocampus are shown in Figure 24 and 25, respectively.

Our results revealed that acute treatment with APAP had no effect on the alterations of synaptic structures in the mPFC and hippocampus. There was no significant difference in all synaptic structural parameters in both brain areas in the rats treated with APAP as compared with the control group (Figure 24-25 and Table 1-2). In the experiment with sub-chronic treatment, the results showed that a significant alteration in the synaptic structures was not observed in the mPFC in the APAP-treated rats, whereas a significantly shorter in the active zone and wider in the synaptic cleft were observed in the hippocampus in rats treated with APAP for 15 days ($P<0.05$ and $P<0.01$, respectively) when compared with those observed in the rats in the control group (Figure 24-25 and Table 1-2). Interestingly, the observation in the rats with chronic treatment demonstrated that rats with the APAP-treatment showed a significant decrease in the length of active zone and increase in width of the synaptic cleft in both the mPFC ($P<0.05$) and hippocampus ($P<0.05$ and $P<0.01$, respectively) when compared with those in the control group (Figure 24-25 and Table 1-2).

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Table 1 The alteration of synaptic ultrastructure in the mPFC of the rats following APAP treatment in three different durations

Synaptic interfacial parameters	Length of active zone (nm)	Width of synaptic cleft (nm)	PSD thickness (nm)	Synaptic curvature
0-day treatment				
Control	439.67±6.55	21.92±0.48	66.61±1.43	0.138±0.009
APAP	428.18±18.96	21.57±0.19	67.17±3.31	0.134±0.009
15-day treatment				
Control	460.65±10.37	22.38±0.36	66.226±2.63	0.140±0.004
APAP	454.42±11.30	21.76±1.04	65.87±0.80	0.130±0.004
30-day treatment				
Control	459.39±14.22	22.93±0.32	62.41±1.64	0.141±0.005
APAP	425.55±6.54 *	24.22±0.27 *	62.21±0.683	0.135±0.009

*P<0.05 compared to the control group

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Table 2 The alteration of synaptic ultrastructure in the hippocampus of the rats following APAP treatment in three different durations

Synaptic interfacial parameters	Length of active zone (nm)	Width of synaptic cleft (nm)	PSD thickness (nm)	Synaptic curvature
0-day treatment				
Control	368.53±20.57	21.37±0.74	64.83±1.25	0.129±0.018
APAP	342.35±17.64	20.50±0.46	61.8±0.84	0.115±0.011
15-day treatment				
Control	346.83±7.63	21.36±0.16	60.20±3.3	0.123±0.007
APAP	316.48±6.99 *	22.90±0.25 **	62.07±3.92	0.112±0.007
30-day treatment				
Control	401.58±27.69	20.49±0.19	61.05±1.19	0.134±0.005
APAP	311.17±11.36 *	21.59±0.20 **	59.01±1.29	0.128±0.009

*P<0.05 and **P<0.01 compared to the control group

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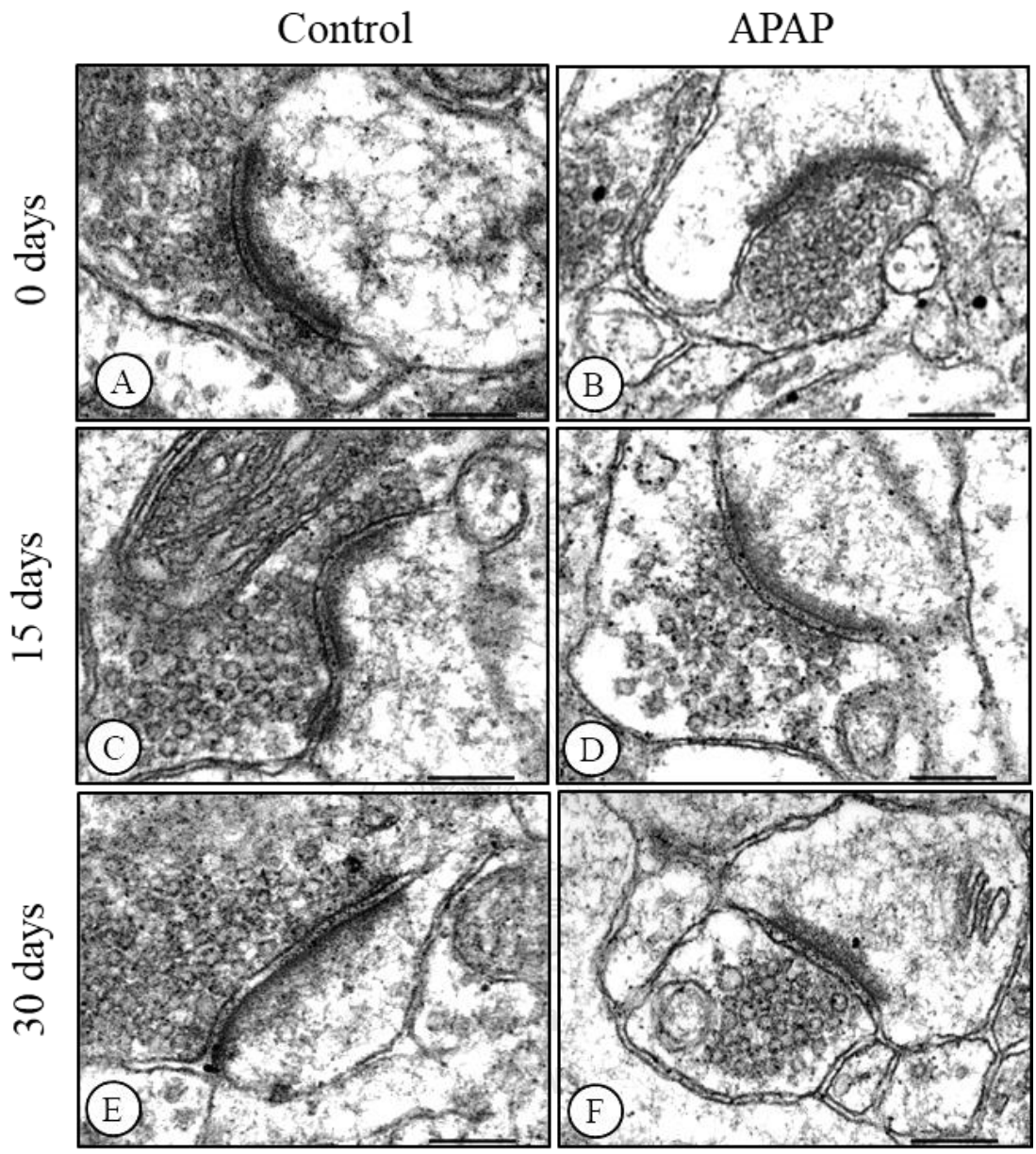

Figure 24 Effect of APAP treatment at the three different durations (0, 15 and 30 days) on the alterations of synaptic ultrastructure in the mPFC. Electron micrographs showing the ultrastructure of synapse obtained from the control (A, C and E) and APAP-treated rats (B, D and F) at the different durations of treatment; scale bar = 200 nm.

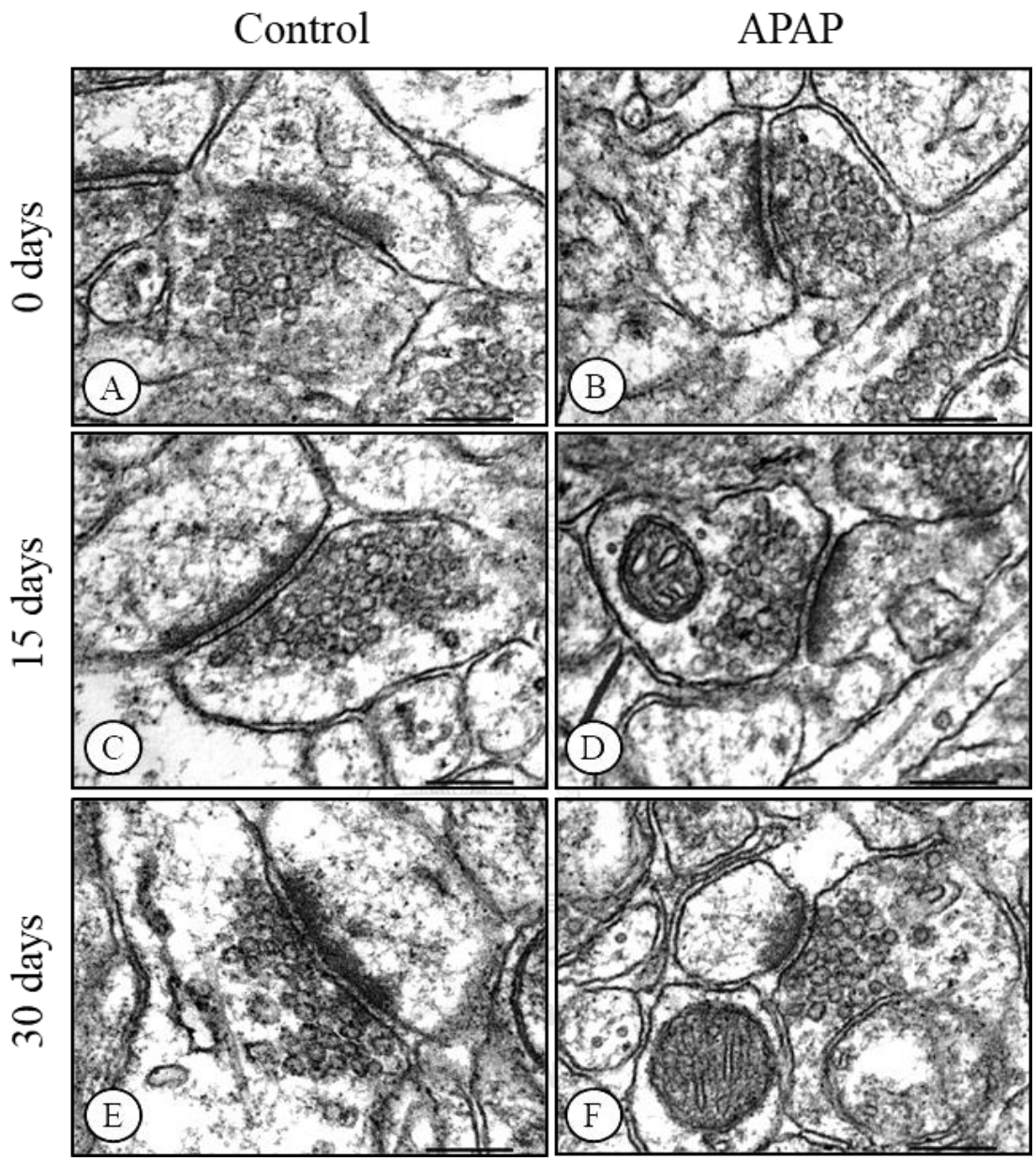

Figure 25 Effect of APAP treatment at the three different durations (0, 15 and 30 days) on the alterations of synaptic ultrastructure in the hippocampus. Electron micrographs showing the ultrastructure of synapses obtained from the control (A, C and E) and APAP-treated rats (B, D and F) at the different durations of treatment; scale bar = 200 nm.

5.2.2 Effect of APAP treatment on the alteration of synaptic proteins

Effect of APAP treatment on the expression of SYP protein

To clarify the effect of chronic APAP treatment on the alteration of learning and memory, the expression of SYP protein (38 kDa) which is a synaptic protein widely used as an indicator for synaptic plasticity was detected in the frontal cortex and hippocampus obtained from all experimental groups.

Using western blot analysis, the results demonstrated that acute APAP treatment had no effect on the expression of SYP protein. There was no difference in the expression of SYP protein in both the frontal cortex and hippocampus of the rats treated with APAP when compared with those to the control group (Figure 26). Our results also showed that while the expression of SYP protein in the frontal cortex was not changed, a significant decrease in this protein was observed in the hippocampus of rats with sub-chronic APAP treatment as compared with that obtained from the control group ($P<0.01$, Figure 26). The results obtained from the experiment of chronic treatment demonstrated that the expression of SYP protein was significantly decreased in both frontal cortex and hippocampus of the rats treated with APAP for 30 days when compared with those to the control group ($P<0.05$ and $P<0.01$, respectively, Figure 26). Furthermore, the multiple comparisons also revealed that the expression of SYP protein in the hippocampus of the rats with sub-chronic (15 days) and chronic (30 days) APAP treatment were as well significantly decreased when compared with the acute APAP-treated rats ($P<0.05$, Figure 26).

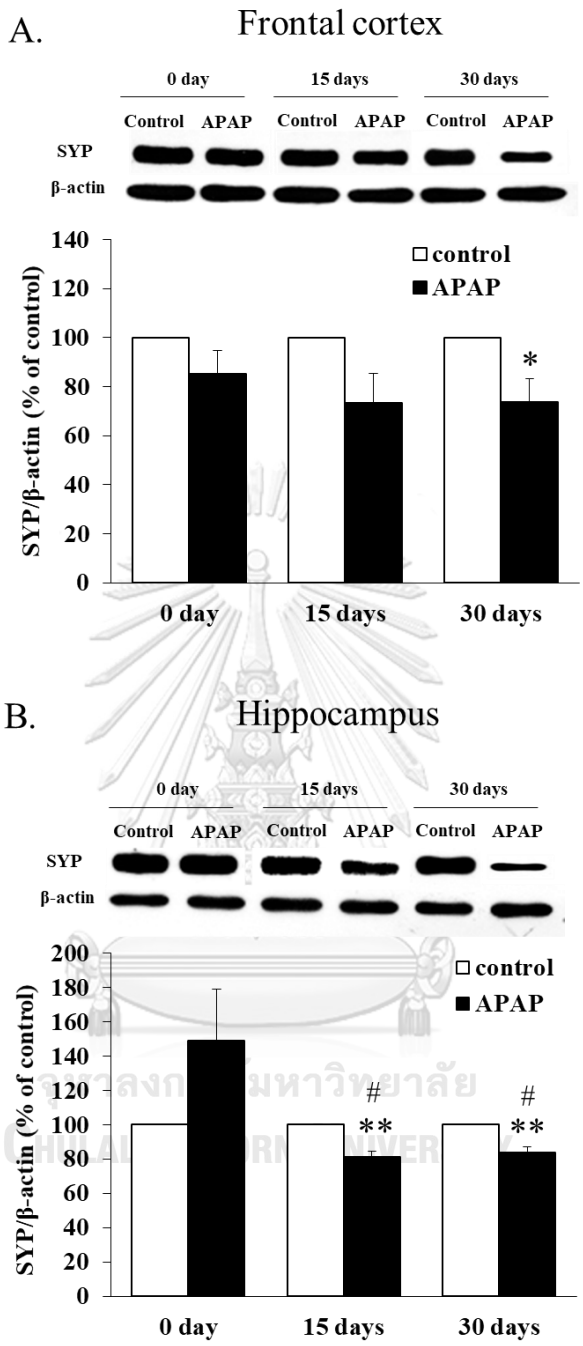

Figure 26 The effect of APAP treatment at the three different durations (0, 15 and 30 days) on the alterations of SYP protein expression. The expression of SYP protein in the frontal cortex (A) and hippocampus (B) obtained from the control and APAP-treated rats. The values are expressed as the mean ± SEM. *P<0.05 and **P<0.01 compared to the control, $^{\#}P$<0.05 compared to the 0-day APAP-treated group.

Effect of APAP treatment on the expression of PSD-95 proteins

In this study, the expression of PSD-95 protein (95 kDa) was used to be an indicator for synaptic plasticity in the brain. Therefore, the expression of PSD-95 protein was monitored in the frontal cortex and hippocampus in all experimental groups.

As compared with the control, the results obtained from western blotting demonstrated no significant difference in the expression of PSD-95 protein in the frontal cortex and hippocampus of the rats with acute APAP treatment (Figure 27). With sub-chronic treatment, our results showed that the expression of PSD-95 protein was not altered in the frontal cortex, whereas a significant reduction of this protein was observed in the hippocampus of the rats treated with APAP for 15 days ($P<0.05$) as compared with the control group (Figure 27). In this study, chronic APAP treatment had no effect on the frontal cortical PSD-95 protein expression. However, a significant decrease in the expression of the hippocampal PSD-95 protein was observed in the rats received chronically with APAP ($P<0.05$) as compared to those from control group (Figure 27). Furthermore, the multiple comparisons also revealed that the expression of PSD-95 protein in the hippocampus of the rats with sub-chronic (15 days) and chronic (30 days) APAP treatment were as well significantly decreased when compared with the acute APAP-treated rats ($P<0.05$ and $P<0.01$, respectively, Figure 27).

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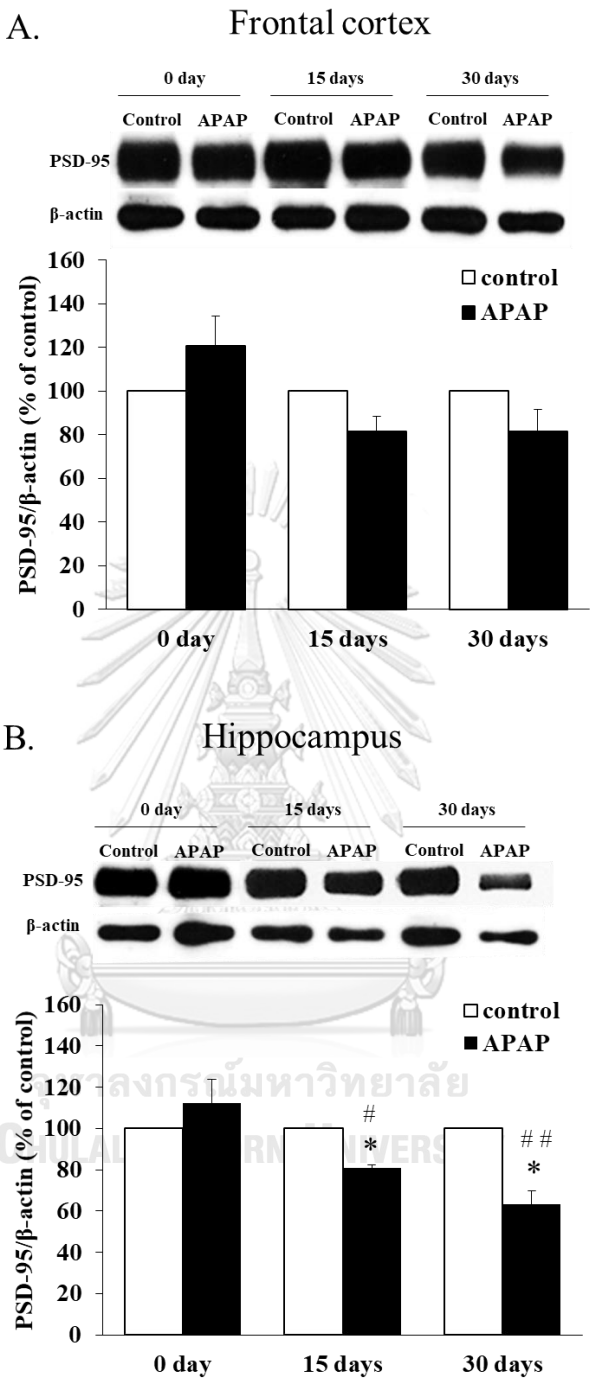

Figure 27 The effect of APAP treatment at the three different durations (0, 15 and 30 days) on the alteration of PSD-95 protein expression. The expression of PSD-95 protein in the frontal cortex (A) and hippocampus (B) obtained from the control and APAP-treated rats. The values are expressed as the mean ± SEM. **P*<0.05 compared to the control, $^{\#}P$<0.05 and $^{\#\#}P$<0.01 compared to the 0-day APAP-treated group.

5.2.3 Effect of APAP treatment on the alteration of BDNF proteins

Using western blot analysis, the expression of BDNF protein was evaluated in both frontal cortex and hippocampus. The results demonstrated that a single dose (0 day) of APAP treatment could not affect the expression of BDNF. After the treatment, the expression of BDNF protein in frontal cortex and hippocampus of the rats treated with APAP was not significantly differenced as compared with those in the control group (Figure 28). The observation in the experiment with sub-chronic treatment demonstrated that the expression of BDNF protein in the hippocampus was significantly decreased in APAP-treated rats as compared with control ($P<0.05$), whereas no different of BDNF expression was observed in frontal cortex (Figure 28). Interestingly, the results obtained from the experiment with chronic treatment showed that treatment with APAP at a long period could induce a decrease in BDNF protein. The expression of frontal cortical and hippocampal BDNF in the rats treated chronically with APAP were significantly lower than that in the control group ($P<0.01$ and $P<0.001$, respectively, Figure 28). Moreover, the multiple comparisons also demonstrated that there was a significant reduction of the BDNF protein in rats treated with APAP for 15 and 30 days when compared with the rats in the acute APAP treatment ($P<0.001$, Figure 28).

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Using immunohistochemistry, the expression of BDNF protein in the mPFC and hippocampus were also monitored. The representative series of the immunohistochemical micrographs in those brain areas were demonstrated in Figure 29 and 30, respectively. With quantitative analysis, there was no statistically significant change in the expression of BDNF in the mPFC and hippocampus, however, the trend for a decrement of BDNF expression in the mPFC and hippocampus was observed in the rats treated chronically with APAP when compared with those to the control (Figure 31).

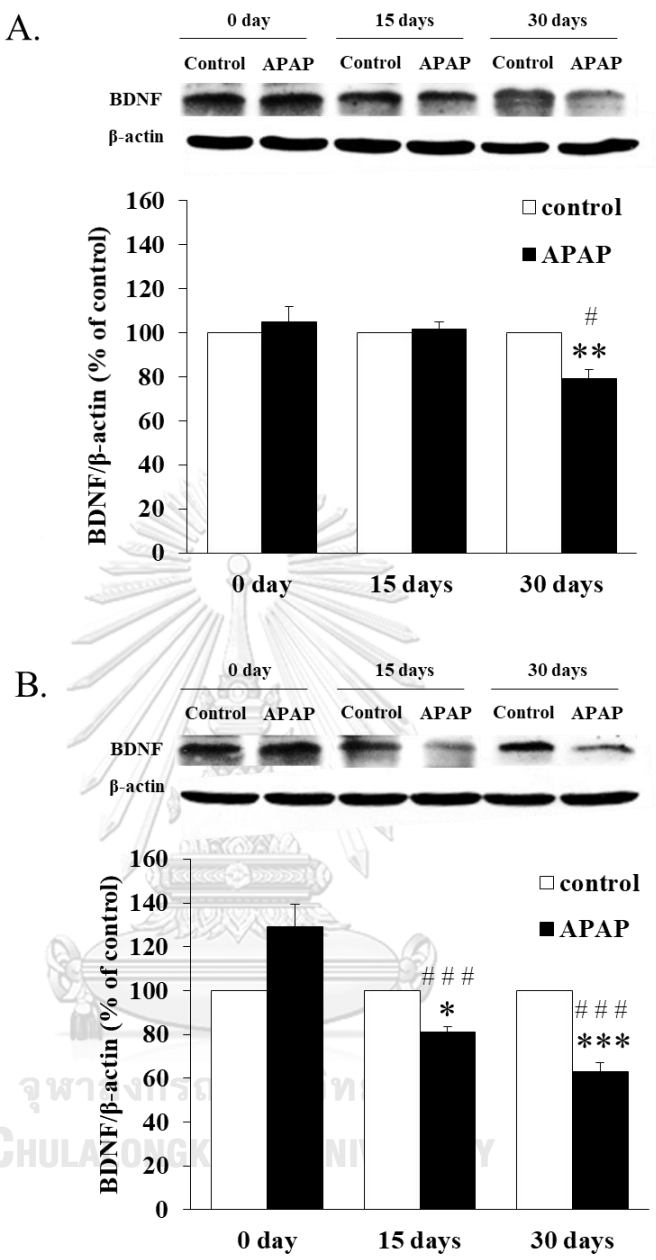

Figure 28 The effect of APAP treatment at three different durations (0, 15 and 30 days) on the alteration of BDNF protein expression. The expression of BDNF protein in the frontal cortex (A) and the hippocampus (B) obtained from the control and APAP-treated rats. The values are expressed as the mean ± SEM. *P<0.05, **P<0.01 and ***P<0.001 compared to the control, $^{\#}P$<0.05 and $^{\#\#\#}P$<0.001 compared to the 0-day APAP-treated group.

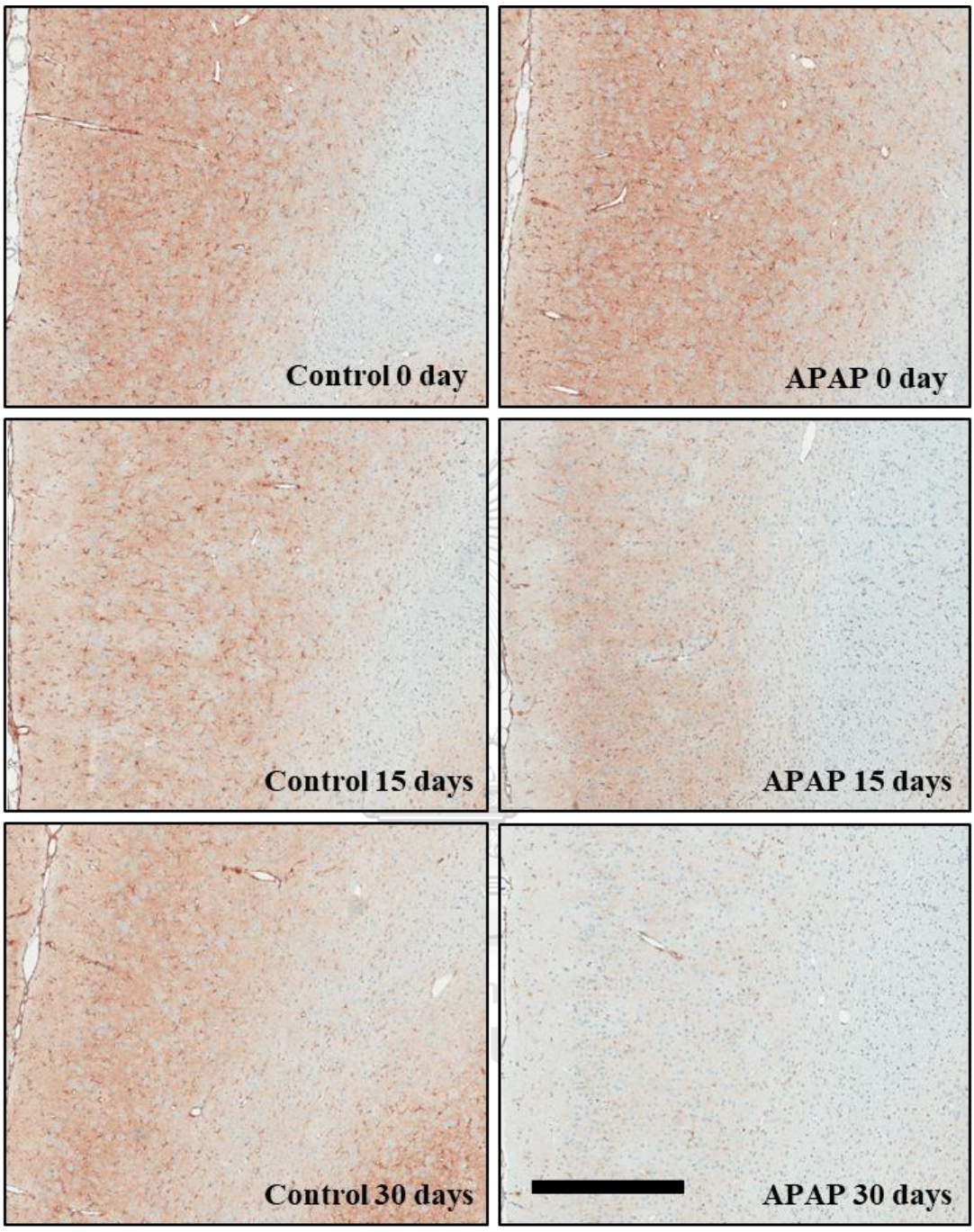

Figure 29 Effects of APAP treatment on at the three different durations (0, 15 and 30 days) on the expression of BDNF protein in the mPFC. Photomicrographs showing BDNF-immunoreactivity (brown color of DAB staining) in the mPFC obtained from the control and APAP-treated rat group at the different durations of treatment; scale bar: 500 μm.

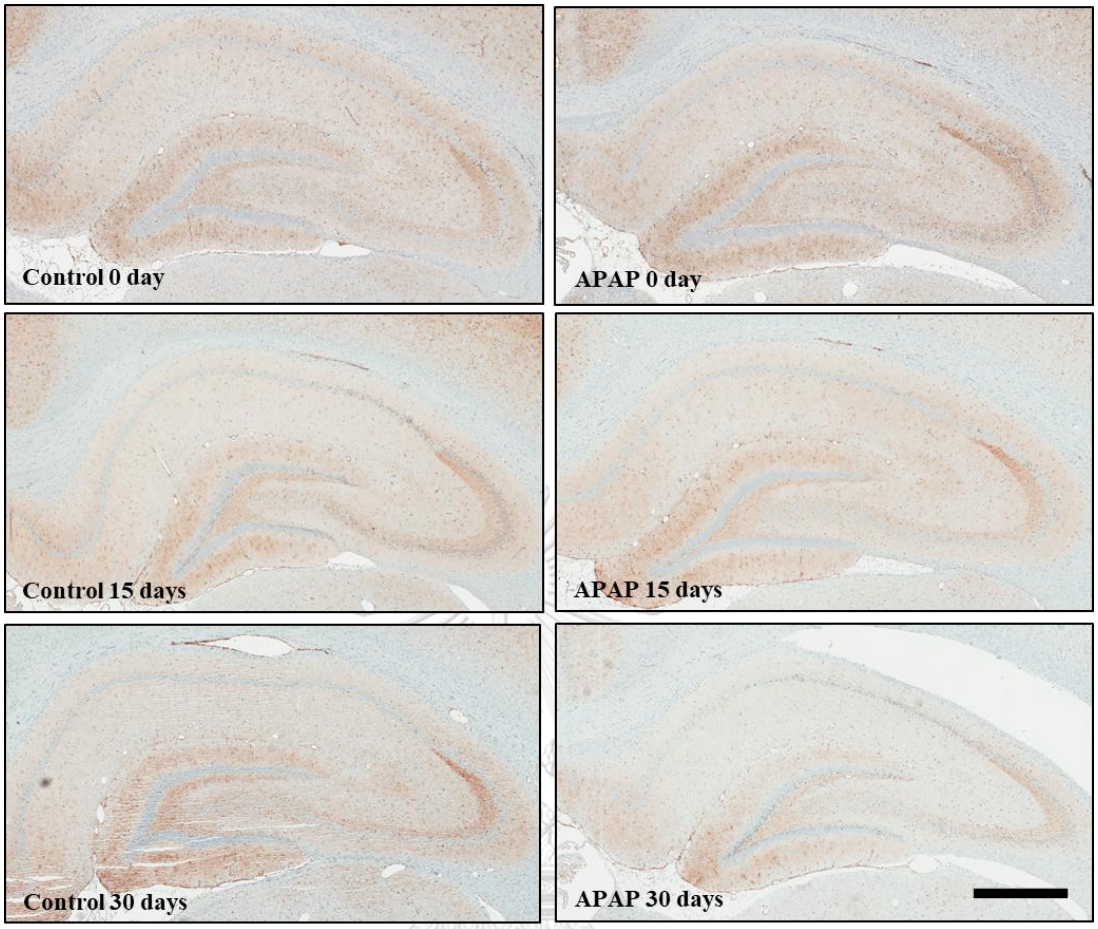

Figure 30 Effects of APAP treatment at the three different durations (0, 15 and 30 days) on the expression of BDNF protein in the rat hippocampus. Photomicrographs showing BDNF-immunoreactivity (brown color of DAB staining) in the hippocampus obtained from the control and APAP-treated rat group at the different durations of treatment; scale bar: 700 μm.

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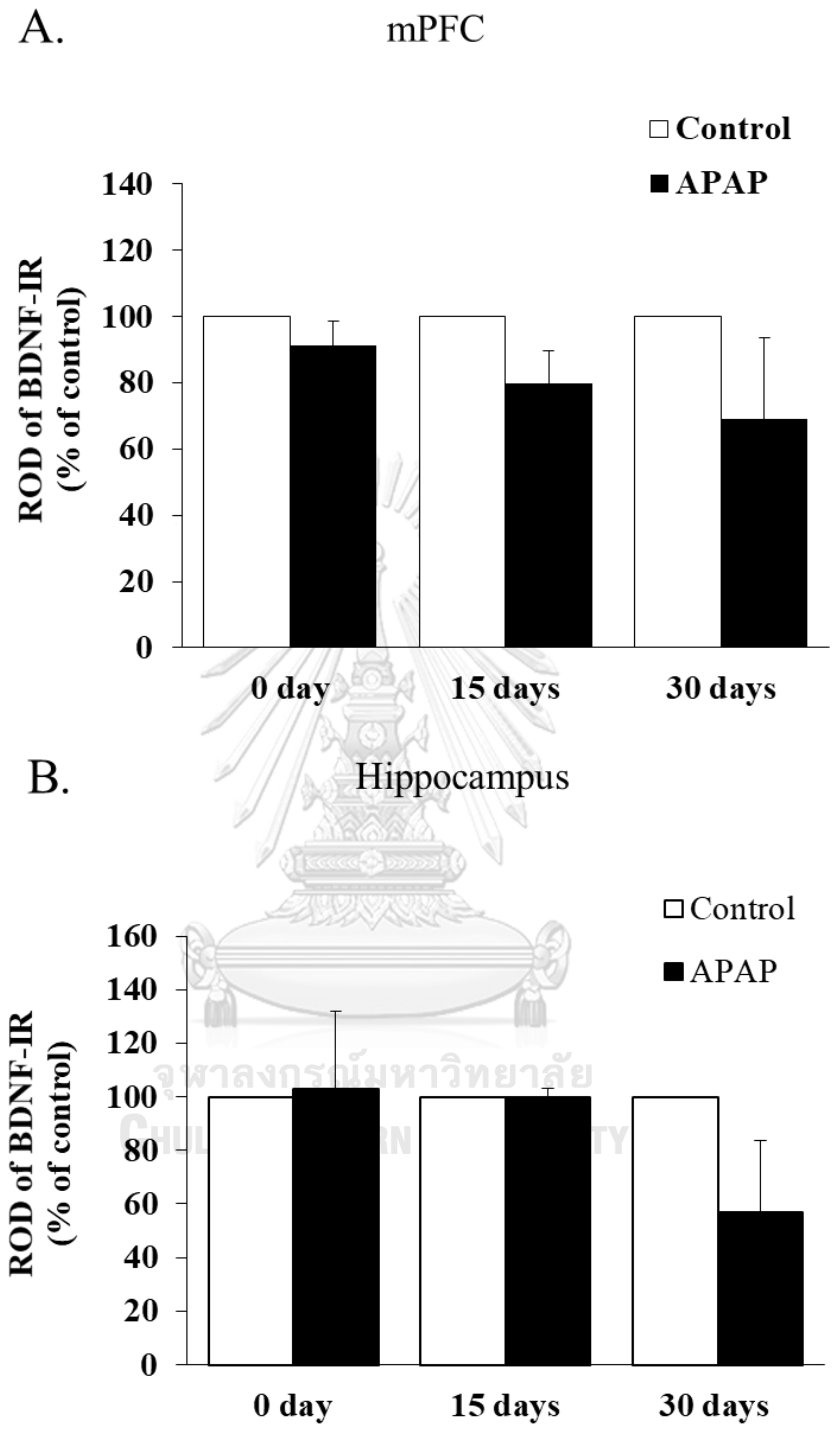

Figure 31 The effect of APAP treatment at the three different durations (0, 15 and 30 days) on the alteration of BDNF protein expression. The immunoreactivity of BDNF in the mPFC (A) and hippocampus (B) obtained from the control and APAP-treated rats by using immunohistochemistry. The values are expressed as the mean ± SEM.

5.2.4 Effect of APAP treatment on the oxidative stress

5.2.4.1 Effect of APAP treatment on the levels of PCO content

In order to investigate whether chronic APAP treatment induces an increase in oxidative stress, the levels of PCO content in both frontal cortex and hippocampus were monitored in all experimental groups. With the DNPH assay, the results demonstrated that acute and sub-chronic APAP treatment had no effect on the levels of PCO content in both the frontal cortex and hippocampus. There was no significant difference in the level of PCO content in 0- and 15-day APAP-treated rats when compared with those of the control group (Figure 32). However, the results obtained from the experiment with chronic treatment demonstrated a significant increase in the levels of PCO content in the hippocampus of the rats treated chronically with APAP as compared to the control. There was no difference in the PCO levels in the frontal cortex obtained from chronic APAP-treated rats as compared with the control group (Figure 32).

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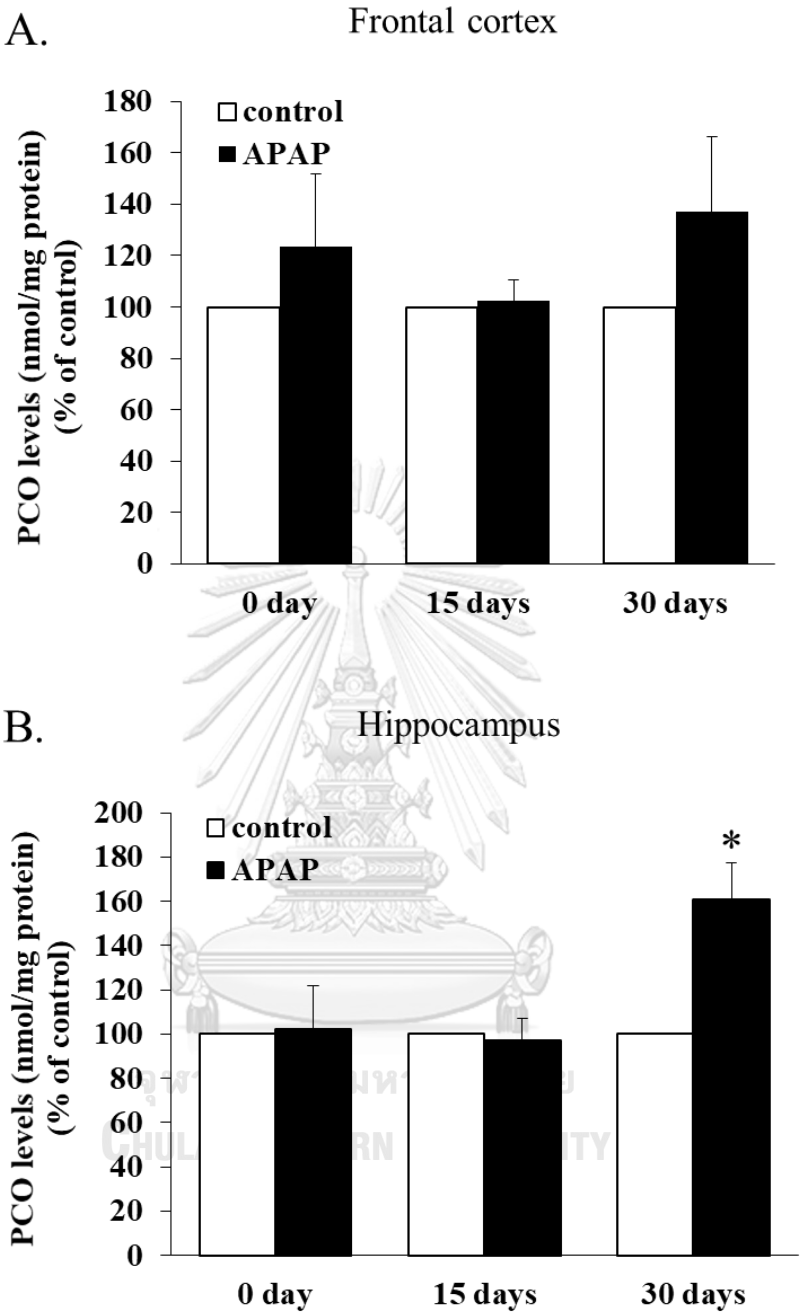

Figure 32 The effect of APAP treatment at the three different durations on the level of PCO content. The levels of PCO in the frontal cortex (A) and hippocampus (B) obtained from the control and APAP-treated rats were evaluated by using DNPH assay. The values are expressed as the mean ± SEM. *P<0.05 compared to the control group.

5.2.4.2 Effect of APAP treatment on the GSH levels

Since GSH is the indicator for antioxidative capacity in the brain, the concentration of GSH in the frontal cortex and hippocampus were evaluated in all experimental groups. In this study, the results revealed that acute and sub-chronic treatment with APAP had no effect on the alterations of GSH levels in rat frontal cortex and hippocampus. A significant difference in the frontal cortical and hippocampal GSH were not observed in the rats treated with APAP for 0 and 15 days when compared to the rats in the control group (Figure 33). A study in the chronic treatment showed that the GSH levels was not changed in the frontal cortex of APAP-treated rats when compared with the control (Figure 33). However, in the hippocampus, the level of GSH was significantly reduced in the rats treated chronically with APAP ($P<0.001$, Figure 33). Moreover, multiple comparisons among the APAP-treated group revealed that the GSH levels in the chronic APAP-treated rats was also significantly decreased when compared to the rats with acute and sub-chronic APAP treatment ($P<0.05$ and $P<0.001$, respectively, Figure 33).

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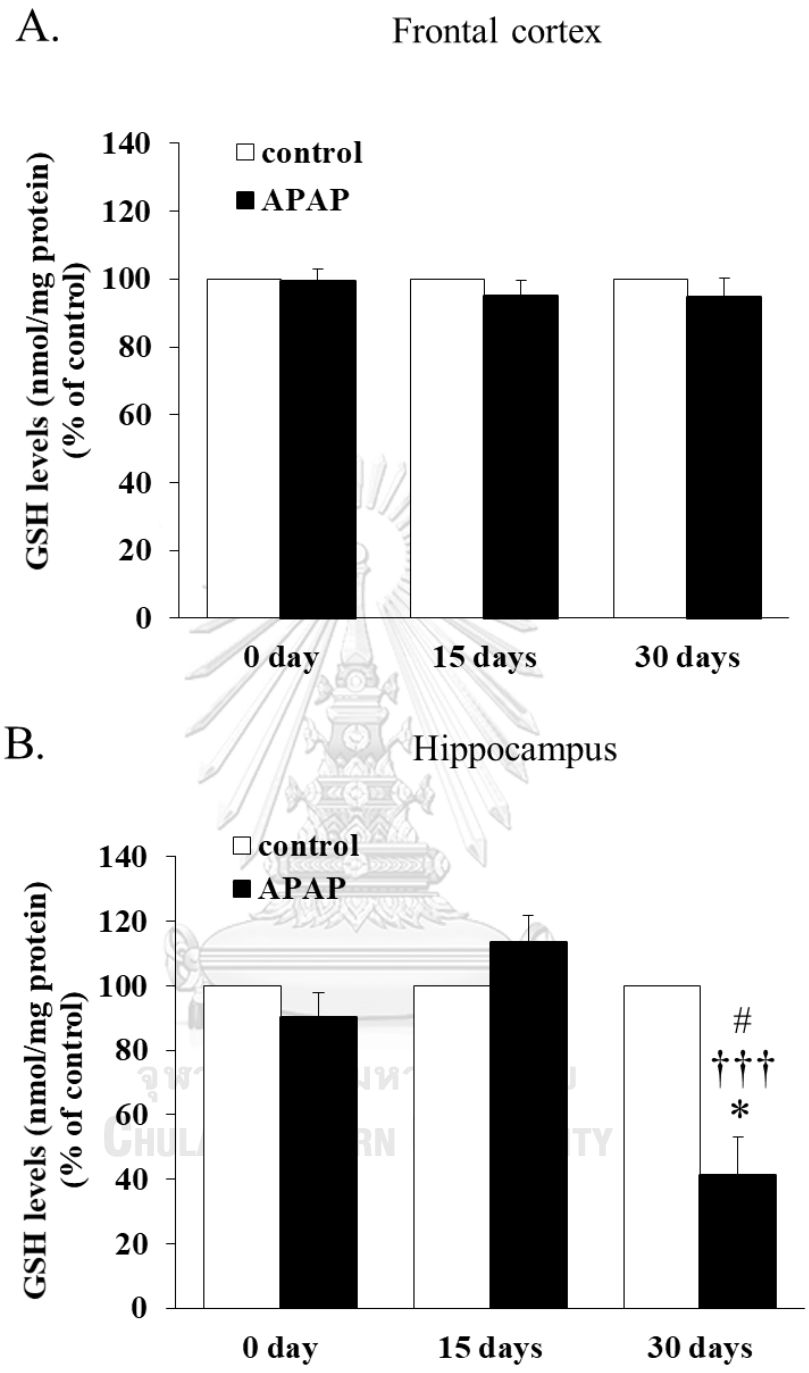

Figure 33 The effect of APAP treatment on the GSH levels. The levels of GSH in the frontal cortex (A) and hippocampus (B) were evaluated by using Cayman's assay kit. The values are expressed as the mean ± SEM. *P<0.05 compared to the control group, $^{\#}P$<0.05 compared to the 0-day APAP-treated rats and $^{\dagger\dagger\dagger}P$<0.001 compared to the 15-day APAP-treated rats.

5.2.4.3 Effect of APAP treatment on the expression of Nrf2 using immunofluorescence staining

With the immunofluorescences staining, the expression of Nrf2 protein in the mPFC and hippocampus were detected in all experimental groups. The results revealed that the Nrf2-immunoreaction could be observed in both cytoplasmic and nuclear compartment of the immunoreactive cells. A study in the 0-day treatment demonstrated that the expression of Nrf2 protein in the mPFC and hippocampus (CA1, CA3 and DG sub-regions) of the APAP-treated rats were not differenced when compared to those observed from the control groups (Figure 34-35). However, treatment with APAP for longer duration demonstrated the different results. The study in the experiment with 15- and 30-day treatment showed that the expression of Nrf2 protein was up-regulated in the rats with APAP treatment. An increment of both cytoplasmic and nuclear Nrf2 in the mPFC and hippocampus (CA1, CA3, and DG sub-regions) were clearly demonstrated in the rats treated with APAP (Figure 34, 36 and 37).

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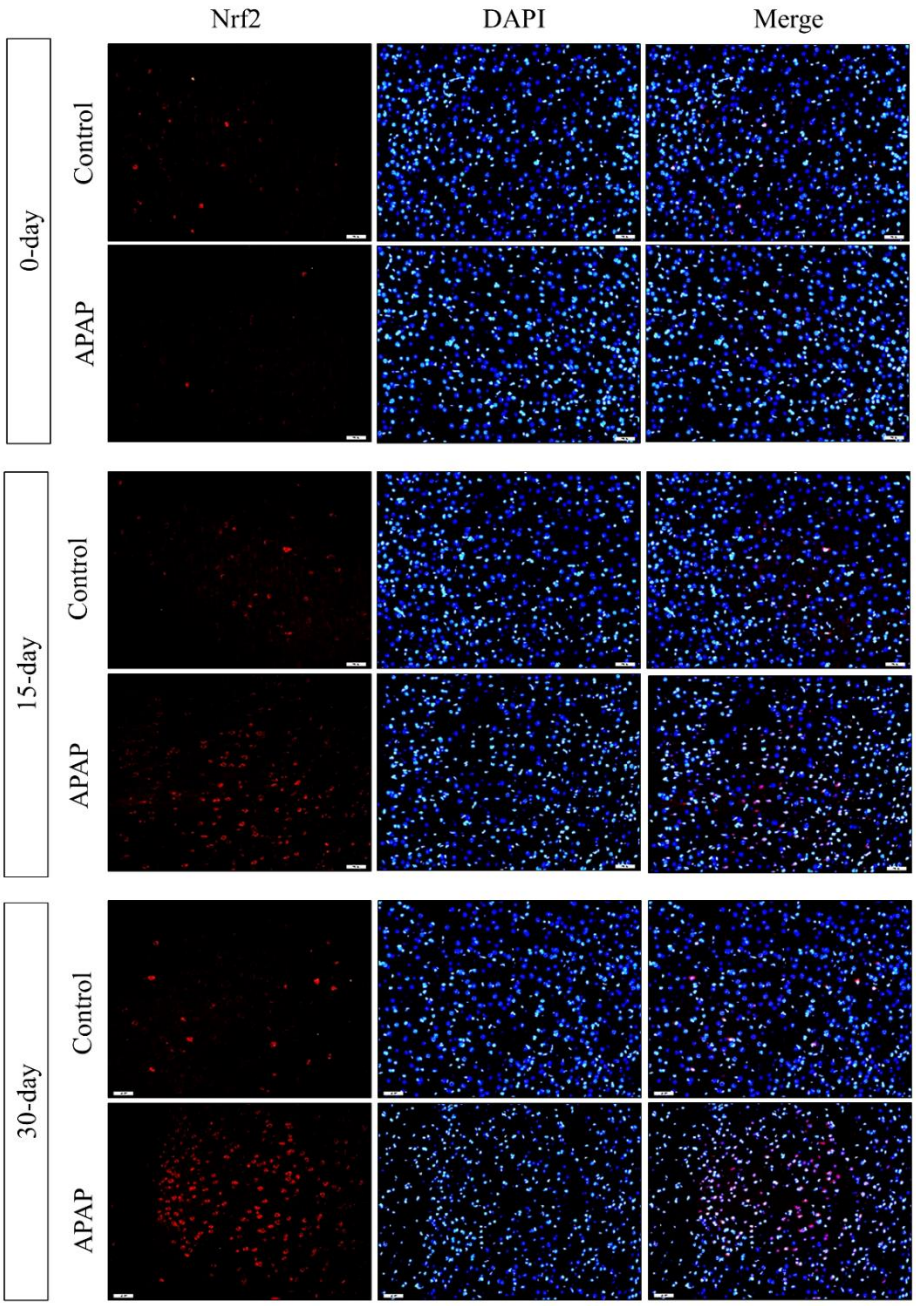

Figure 34 Effect of APAP treatment at the three different durations (0, 15 and 30 days) on the expression of Nrf2 protein in the rat mPFC. Representative images of immunofluorescence staining of Nrf2 (red) in the mPFC obtained from the control and APAP-treated rat groups; scale bar: 50 μm.

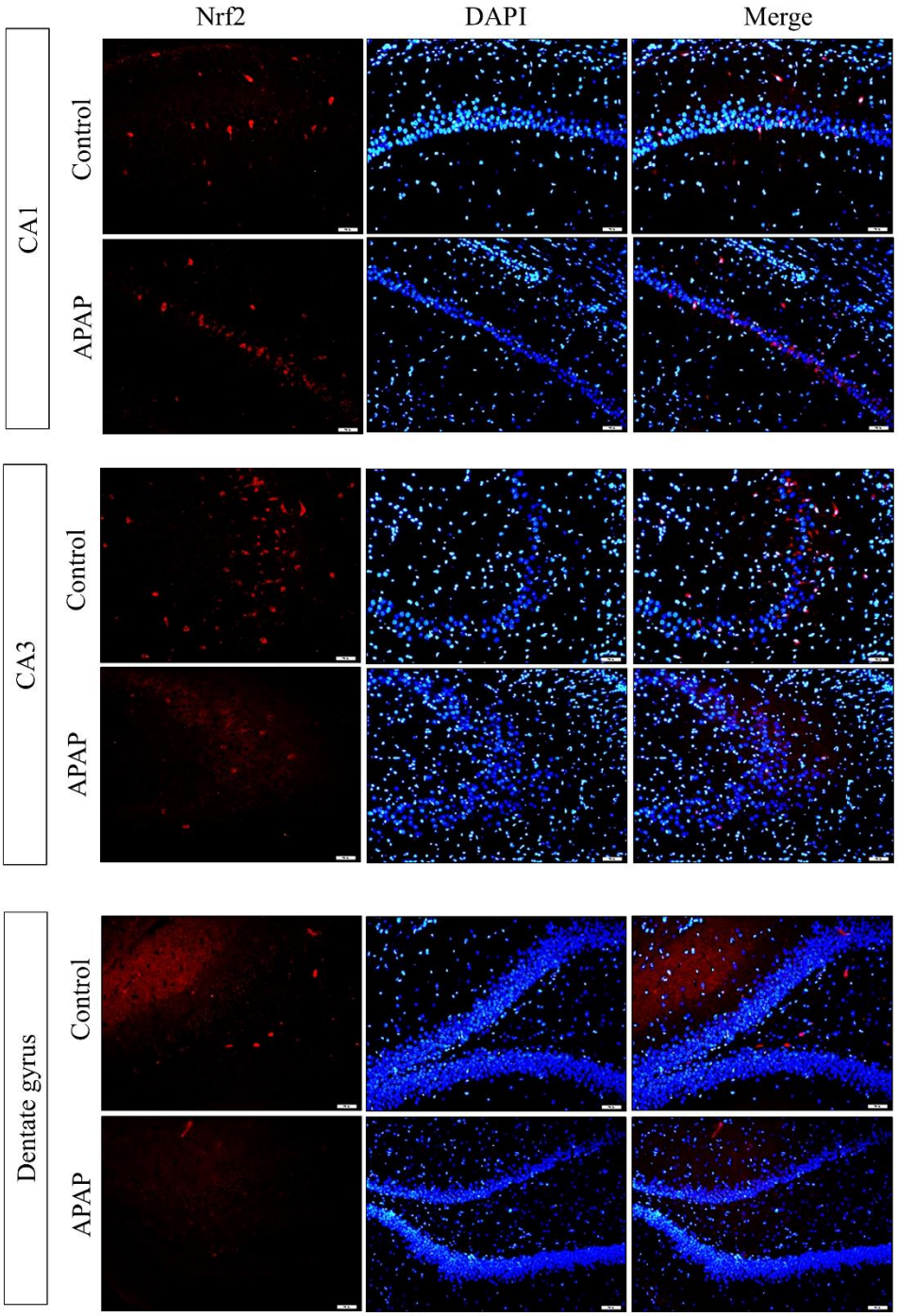

Figure 35 Effect of 0-day APAP treatment on the expression of Nrf2 protein in the hippocampus. Representative images of immunofluorescence staining of Nrf2 (red) in the CA1, CA3 and DG sub-regions of the hippocampus obtained from the control and APAP-treated rat groups; scale bar: 50 μm.

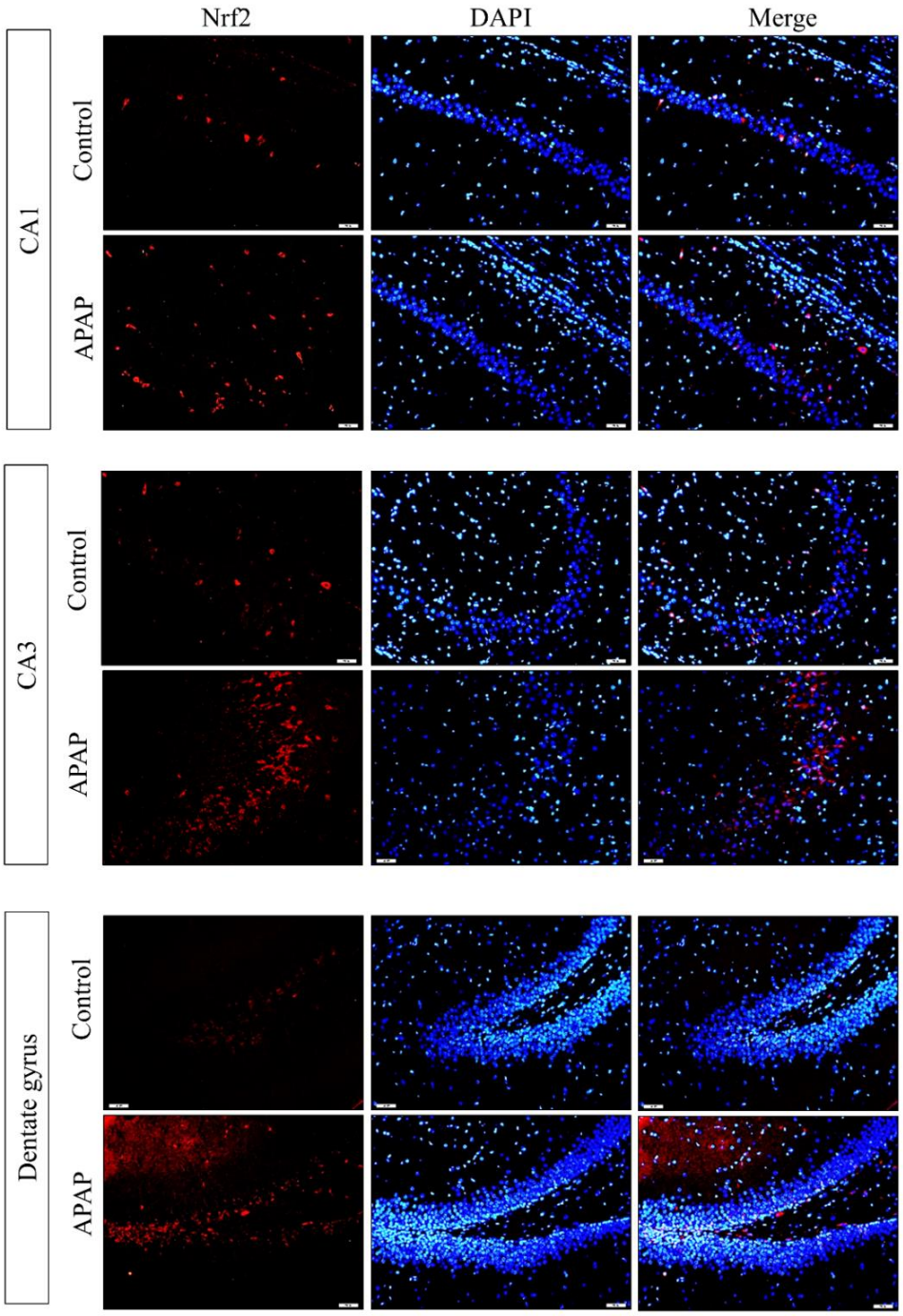

Figure 36 Effect of 15-day APAP treatment on the expression of Nrf2 protein in the hippocampus. Representative images of immunofluorescence staining of Nrf2 (red) in the CA1, CA3 and DG regions of the hippocampus obtained from the control and APAP-treated rat groups; scale bar: 50 μm.

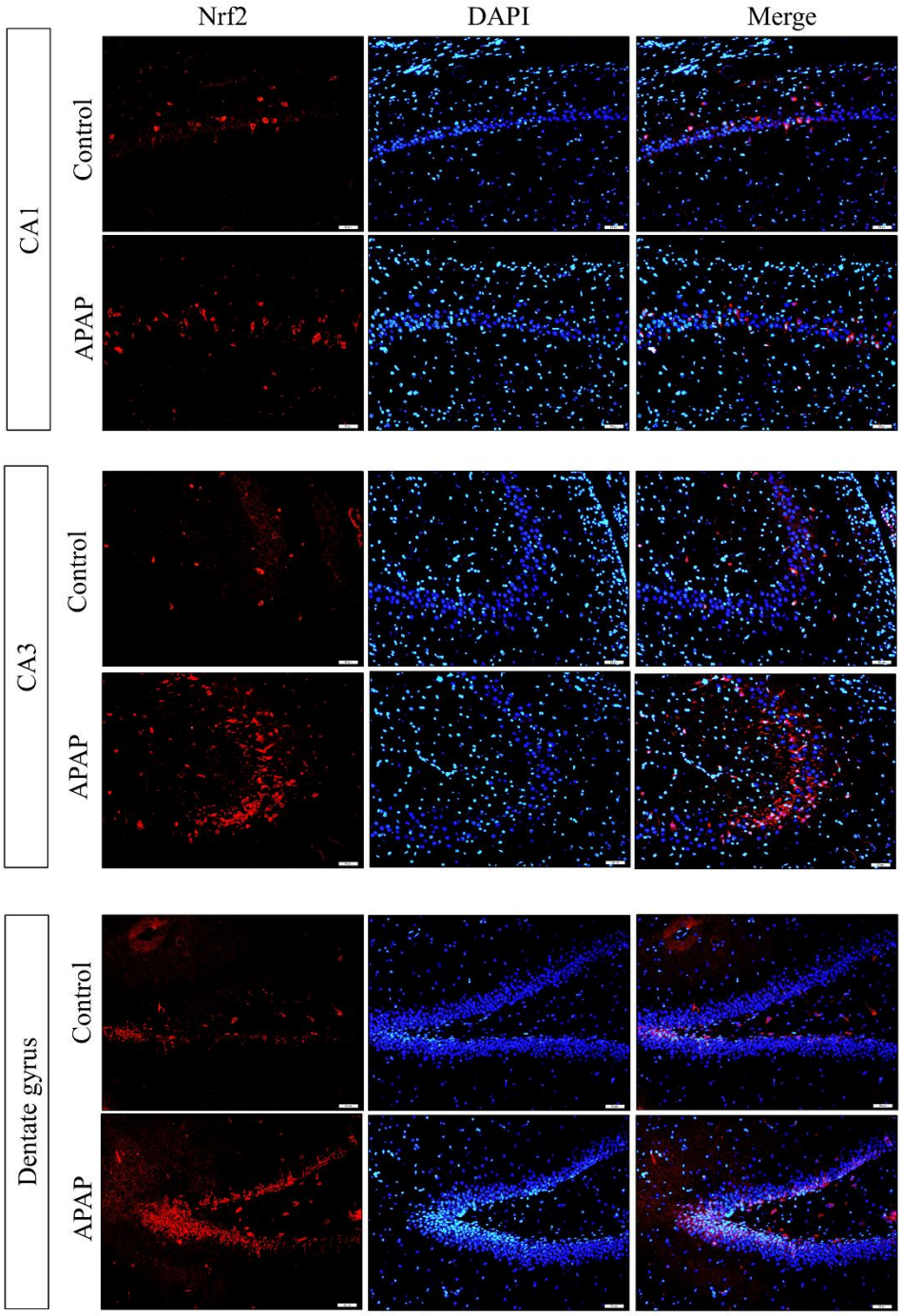

Figure 37 Effect of 30-day APAP treatment on the expression of Nrf2 protein in the hippocampus. Representative images of immunofluorescence staining of Nrf2 (red) in the CA1, CA3 and DG regions of the hippocampus obtained from the control and APAP-treated rat groups; scale bar: 50 μm.

CHAPTER V

DISCUSSION

Regarding the effects of APAP treatment on the CNS, several research groups have demonstrated the impact of this drug treatment on learning and memory. However, the exact effect of this drug exposure on cognitive outcome has not been concluded to date. The results observed in the present study indicate the different effects of the acute and chronic APAP treatment on the learning and memory. We demonstrated that acute treatment with APAP had no any effect on learning and memory process. Whereas, exposure to this drug for the longer period (more than 2 weeks) could induce an impairment of learning and memory. Also, the alterations of learning and memory behavior found in the prolonged APAP treatment were accompanied with an abnormality of the synaptic integrity, a down-regulation of BDNF protein and an increase in oxidative stress.

According to the dose of APAP employed in this study, daily treatment with APAP 200 mg/kg bw in the rat is equal to the 32.25 mg/kg bw in human based on the conversion of the animal dose to human equivalent dose (http://www.fda.gov/ucm/groups/fdagov-public/@fdagov-drugs-gen/documents/document/ucm078932.pdf; accessed December 16, 2018). Therefore, the dose of APAP used in the present study (200 mg/kg bw) is considered to be the dose within the recommended dose range for APAP treatment.

The present results showed that acute treatment with 200 mg/kg bw APAP had no effect on learning and memory since the NOR performance of the rats treated with a single dose of APAP was not different from the rats in the control groups. Moreover, the results obtained from both the biochemical and ultrastructural studies have correspondingly confirmed that using this drug for acute treatment has

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no side effect on learning and memory since there was no changing in the synaptic integrity (synaptic ultrastructure and proteins), the expression of BDNF protein and oxidative stress in both the frontal cortex and hippocampus in APAP-treated group.

As mentioned earlier, APAP can easily pass through the BBB. After reaching the cerebral circulation, therefore, APAP can quickly distribute throughout the brain and metabolized by the CYP2E1 enzyme which results in the generation of NAQPI. After forming, NAPQI is rapidly counteracted with exist GSH to neutralize its toxicity [9, 83, 136, 137]. In the case of excessive generation of NAPQI, this toxic molecule can directly bind to cellular proteins causing cell damage and death [150]. In this study, the level of protein oxidation and GSH in the frontal cortex and hippocampus obtained from the rats with acute treatment of APAP were not different from the control group suggesting no oxidative stress formation in those brain areas. Based on these results, it is possible that, with acute treatment, NAPQI can be completely captured by existing GSH in the cells. For this circumstance, the homeostasis in the brain is not disturbed therefore there is no change in the synaptic plasticity and learning and memory performance. Our findings in this part indicate that short-term treatment with APAP does not harm cognitive memory abilities, which is well correlated with a previous document claiming that APAP is a safe drug when used within a therapeutic dose for a short period (https://www.fda.gov/Drugs/DrugSafety/ucm239821.htm; accessed December 30, 2018).

Interestingly, exposure to APAP for the longer period (15 and 30 days) revealed the different results. With sub-chronic treatment, a decrement of NOR and MWM performances were demonstrated in the rats with APAP exposure. Moreover, the results from the ultrastructural study in the hippocampus demonstrated an

abnormality of synaptic plasticity (a shortening of the active zone and widening of the synaptic cleft) in the APAP-treated group.

It is known that the active zone is the pre-synaptic membrane compartment indicating the operative area of synaptic contact and neurotransmitter release [38]. Regarding the alteration of the synaptic cleft, the association between the synaptic clef widening and the decrement of signal transduction at the synapses is well recognized [40]. As we know that an alteration of synaptic ultrastructure can result in the changing of synaptic transmission at the synapses, in this case, the ultrastructural changes of the synapses observed in the rats with sub-chronic APAP treatment can indicate an abnormality of synaptic plasticity in this group.

The abnormality of the synaptic plasticity in the rats with sub-chronic APAP treatment was confirmed with the results from western blot analysis which demonstrated the reduction of both the SYP and PSD-95 protein expression in the hippocampus of the rats with 15-day APAP treatment. It is well recognized that both SYP and PSD-95 are the synapse-associated proteins involved in the neurotransmission at the synapse. SYP is a pre-synaptic membrane protein present abundantly in synaptic vesicles [54]. This protein plays an important role in the regulation of moving, docking and neurotransmitter releasing of synaptic vesicles [47, 56, 57]. At the post-synaptic site, PSD-95 is the most abundant scaffolding protein which lies beneath the postsynaptic membrane. This protein has been proved to associated with synaptic plasticity due to its property which involves in several synaptic processes including clustering of the receptors and regulation of the receptor functions [65]. Depletion of both SYP and PSD-95 proteins have been reported to correlate with a decrease of synaptic connections at the synapses and decline in the cognitive function [73]. It is as well proposed that changing in the synaptic interfaces can be one indicator for the early stage of learning and memory

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deficit [50]. Therefore, the alteration of the synaptic ultrastructure and proteins observed in the rats with sub-chronic APAP treatment can be the reason explaining the reduction of learning and memory performance observed in these rats.

The presented results are in accordance with the findings in recent studies which demonstrated that prolonged treatment with APAP can induce an adverse effect on the cognitive function. In 2013, the Norwegian sibling control study established that the children who had maternal long-term exposure to APAP showed a higher risk for ADHD and HKD [18]. A recent study in an animal experiment has also demonstrated that early exposure to APAP for a long period could reduce social behaviors and recognition memory [151]. Furthermore, the same group of investigators has also provided evidence that treatment with APAP for 8 weeks even in the therapeutic dose range (10 and 50 mg/kg bw) could impair several amino acids profile in many brain regions including the hypothalamus, striatum and mPFC which can expect for impairment of learning and memory [132].

The effect of APAP treatment on the alteration of learning and memory is clearly demonstrated by the results obtained from the rats with the chronic APAP treatment. The results showed that 30-day treatment with APAP could reduce NOR and MWM performances indicating an impairment of learning and memory in these rats.

Regarding the results obtained from electron microscopic study, the ultrastructural alterations indicating synaptic degeneration have been clearly observed in rats with chronic (30 days) APAP treatment. Not only change in the synaptic interfaces (decrease in the length of the active zone and the increase in width of the synaptic cleft) but also decrease in numbers of the synapses in both the frontal cortex and hippocampus of the rats with chronic APAP treatment.

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It is well accepted that an alteration of the synaptic density can reflect the capacity of neurotransmission at the synapses [44, 45]. A loss in the synaptic density is well documented to be tightly associated with the cognitive impairment in several neurodegenerative disorders [44, 45]. Thus, the alteration of the synaptic ultrastructure observed in the rats with chronic APAP treatment indicates a diminishing of the synaptic transmission in both the frontal cortex and hippocampus of these rats.

An abnormality of synaptic transmission following prolonged APAP exposure has further been confirmed by the results from western blot analysis which demonstrated the down-regulation of synapse-related proteins (SYP and PSD -95) in the frontal cortex and hippocampus obtained from the rats treated chronically with APAP. Taken the demonstration of ultrastructural abnormality of the synapses together with the down-regulation of synaptic proteins observed in rats treated with APAP for long period, it can be ensured that treatment with APAP for a long duration can induce an abnormality in synaptic transmission in both the frontal cortex and hippocampus. These present results are important evidence supporting the findings from previous reports indicating the deleterious effect of prolonged APAP treatment on learning and memory [18, 132].

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BDNF is widely known as a growth factor which plays a key role in several neuronal activities, including synaptic plasticity [152, 153]. The role of this protein in learning and memory processes has as well been accepted. Among all brain regions, the distribution of BDNF and its major receptor, tyrosine receptor kinase B (TrkB), are highest in the cerebral cortex and hippocampus [75, 76]. The deletion of this growth factor is reported to result in cognitive impairment [154, 155], whereas injection with exogenous BDNF can enhance learning and memory in animals [156]. This cumulative evidence suggests a potential influence of BDNF on learning and memory function. In

our present study, in parallel with the reduction of behavioral performance, the down-regulation of BDNF protein in both the frontal cortex and hippocampus were clearly observed in the rats with long term APAP treatment. These findings correlate with the results observed in a recent study. In 2018, Blecharz-Klin et al. have revealed that prolonged treatment with APAP (prenatal treatment) could impair cognitive function and this deleterious effect was well correlated with a reduction of striatal BDNF protein [151].

The previous study has suggested that BDNF can facilitate the synaptic activity associated with learning and memory via modulating the synaptic proteins and their elements [26]. The findings obtained our study demonstrated a reduction of frontal cortical and hippocampal synapse-related proteins (SYP and PSD-95) in the 30-day APAP-treated rats. With these results, it can be suggested that the down-regulation of BDNF in the frontal cortex and hippocampus following long-term APAP treatment is at least one mechanism underlying synaptic degenerative effect of chronic APAP treatment and this phenominon can further result in learning and memory impairment.

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Moreover, it is widely accepted that oxidative stress has a negative relationship to learning and memory [26, 157, 158]. Depletion of energy, DNA damage, induction of neuroinflammation and apoptosis are proposed to be the mechanisms underlying the memory deficit induced by oxidative stress [159, 160]. In this study, an increase of oxidative stress is as well demonstrated in the rats with chronic APAP treatment. An elevation of the PCO level and depletion of GSH in the brain obtained from the rats with 30-day APAP treatment were demonstrated. Apart from the direct observation of protein oxidation, an alteration in the expression of the antioxidant associated protein, Nrf2, was as well monitored in this study.

Nrf2 is a transcription factor which is a potent regulator of many antioxidant genes expression in the cells including the GSH-related gene transcription [94, 103]. Although the role of Nrf2 in various neuropathological conditions is still not fully understood, it was proposed that the fluctuation of Nrf2 expression might be manipulated by the disease mechanisms [108]. For example, decreased Nrf2 level was demonstrated in AD patient despite the presence of oxidative stress [105], whereas an increase in the expression of the ARE-related genes was observed in patients with mild cognitive impairment [106, 107]. In the present study, an up-regulation of Nrf2 was well demonstrated in long-term APAP treatment. With these results, it can be assumed that increased Nrf2 expression can indicate the neuronal response against oxidative damage through prolonged APAP exposure.

Different from a short-term treatment, chronic APAP treatment can result in the continuous generation of NAPQI in the brain which cannot be completely detoxified with exist GSH in the cells. This event can finally result in the depletion of GSH level and accumulation of NAPQI in those brain areas. As we know that NAPQI is extremely toxic to the cells, it can destruct several functional molecules including synapse-associated proteins. Additionally, depletion of GSH can lead to an increase in oxidative stress which known to be associated with an alteration of synaptic plasticity [161]. Several previous studies have revealed that an increase in oxidative stress is well interrelated with the reduction of synaptic-associated proteins (synapsin-I, SYP, PSD-95 and SNARE proteins) and alteration of synaptic ultrastructure in the hippocampus [26, 162]. Furthermore, previous studies have demonstrated that an increase in oxidative stress could manipulate the expression of BDNF [26, 91]. Although the action of oxidative stress interfering the expression of BDNF is very complicated, it is suggested that several factors might be involved. It has been proposed that an elevation of oxidative stress could decrease the DNA-binding

activities of the activator protein-1, which can reduce the BDNF gene expression [91]. Some investigators have also suggested that oxidative stress could disrupt the function of NMDA channel due to energy depletion [163, 164], which would result in the reduction of BDNF gene expression [92, 95]. Based on these accumulative data, we suggest that an increase in protein oxidation induced by oxidative stress in the brain following chronic treatment with APAP is involved in the alteration of synaptic integrity and BDNF expression in the frontal cortex and hippocampus and can finally lead to an abnormality of learning and memory.

With regard to the findings observed in this study, the effect of APAP treatment on the alteration of learning and memory can be summarized as demonstrated in the diagram in Figure 38.

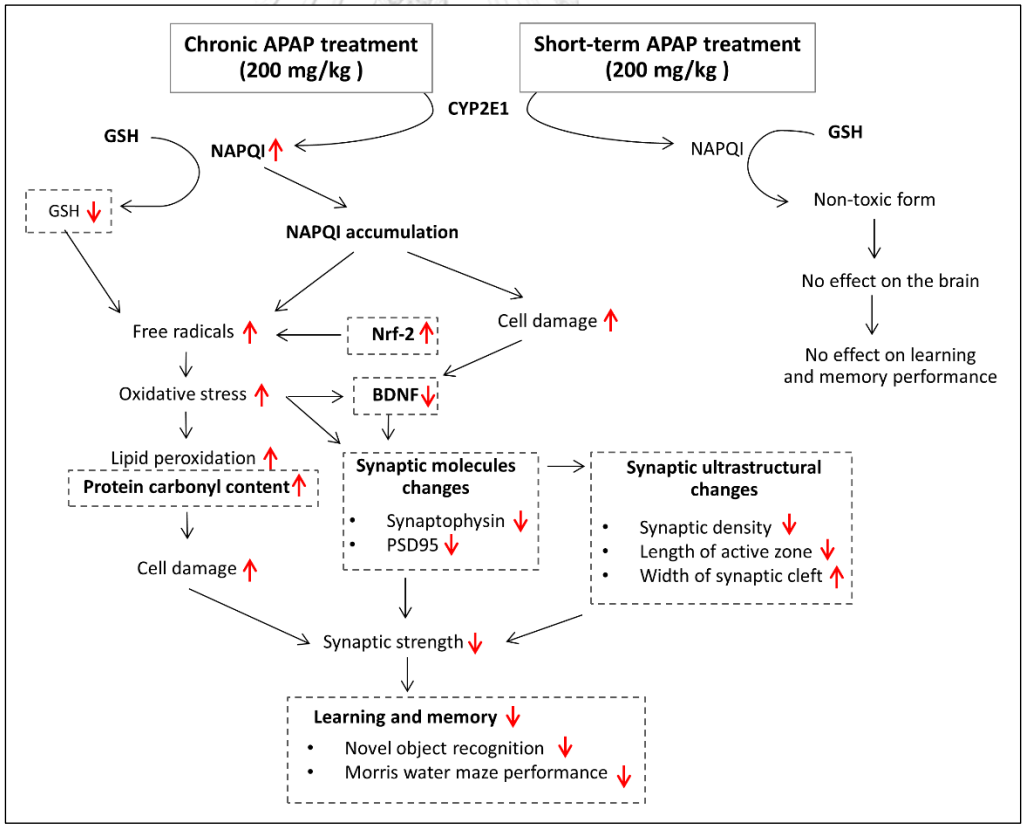

Figure 38 Diagram explaining the effect of APAP treatment on the alteration of learning and memory

In conclusion, the results obtained from the present study are one more evidence supporting that APAP can still be recognized as a safe drug when used for a short period. However, exposure to this drug for a long period even with the dose within the therapeutic range can possibly disturb the learning and memory function. Whether these findings can reflect the effect of this drug treatment in human still needs to be further explored.

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APPENDIX

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Reagent preparation

10X SDS-PAGE running buffer, final volume 1 liter (250 mM Tris-HCl, 1.92 M Glycine, 1% SDS, pH 8.3)

- Tris-base (MW 121.14) 30.28 g
- Glycine (MW) 75.07) 144.13 g
- Sodium dodecyl sulfate (SDS) 10 g

  Add H_2O up to 1000 ml

10X Transfer buffer, final volume 800 ml: (250 mM Tris-HCl, 1.92 M Glycine, 0.025% SDS, pH 8.3)

- Tris-base (MW 121.14) 30.28 g
- Glycine (MW 75.07) 144.13 g

  Add dH_2O up to 800 ml

  Store at room temperature

10X Tris-buffer saline (TBS), final volume 1 liter: (200 mM Tris, 1.5 M NaCl, pH 7.6)

- Tris-base (MW 121.14) 24.23 g
- NaCl (MW 58.4) 80.06 g

  Add dH_2O almost ~ 800 ml → adjust pH to 7.6 by HCl

  Add dH_2O up to 1000 ml

  Store at room temperature

10X TBS buffer, final volume 1 liter: (50 mM Tris, 150 mM NaCl, pH 7.6)

- Tris-base (MW 121.14) 60.57 g
- NaCl (MW 58.4) 87.6 g

  Add dH_2O up to 800 ml → adjust pH to 7.6 by HCl

  Add dH_2O up to 1000 ml

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Store at room temperature

10% SDS (W/V) final volume 100 ml:

- SDS 10 g

  Add dH2O up to 100 ml

  Store at room temperature

4X Running gel buffer, final volume 200 ml: (1.5 M Tris-HCl, pH 8.8)

- Tris-base (MW 121.14) 36.3 g

  Add dH_2O almost ~ 150 ml → adjust pH to 8.8 by HCl

  Add dH_2O up to 200 ml

  Store at 4 ℃

4X SDS Protein Sample Buffer (4x Loading dye), final volume 10 ml: (240 mM Tris-HCl (pH 6.8), 40% Glycerol, 8% SDS, 0.04% Bromophenol blue, 5% β-mercaptoethanol)

- 1 M Tris-base (pH 6.8) 2.4 ml
- 100% Glycerol 4 ml
- SDS 0.8 g
- 1% Bromophenol blue 0.4 ml
- β-mercaptoethanol 0.5 ml

  Add dH_2O 2.7 ml

  Store at -20 ℃

Coomassie blue staining, final volume 1 liter: (0.1% (w/v) Coomassie Brilliant Blue R250, 40% Methanol, 10% Glacial acetic acid)

- Coomassie Brilliant Blue R250 1 g
- Methanol 400 ml

  Stir ~ 3 hours until dissolved. Then add:

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- Glacial acetic acid 100 ml

  Add dH_2O up to 1000 ml *filter before store*

  Store at room temperature

Destaining solution I, final volume 1 liter: (40% Methanol, 10% Glacial acetic acid)

- Methanol 400 ml
- Glacial acetic acid 100 ml

  Add dH_2O up to 1000 ml

  Store at room temperature

Destaining solution II, final volume 1 liter: (10% Methanol, 5% Glacial acetic acid)

- Methanol 100 ml
- Glacial acetic acid 50 ml

  Add dH_2O up to 1000 ml

  Store at room temperature

Ice-cold Tris buffer for washing specimen before extracting, final volume 500 ml: (5mM Tris-HCl, pH 7.4, autoclave)

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- Tris-base (MW 121.14) 0.3 ml

  Add dH_2O almost ~ 400 ml → adjust pH to 7.4 by HCl

  Add dH_2O up to 500 ml

  Store at 4 °C

SDS-PAGE

Stacking gel (for 2 gels)				
30% Acrylamide			0.44 ml	
4X Stacking gel buffer			0.83 ml	
10% SDS			33 µl	
dH_2O			2.03 ml	
10% Ammonium persulfate			25 µl	
TEMED			3 µl	
Separating gel (for 2 gels)	**7.5%**	**10%**	**12.5%**	**15%**
30% Acrylamide	2.5 ml	3.3 ml	4.2 ml	5 ml
4X Stacking gel buffer	2.5 ml	2.5 ml	2.5 ml	2.5 ml
10% SDS	100 µl	100 µl	100 µl	100 µl
dH_2O	4.9 ml	4.0 ml	3.2 ml	2.4 ml
10% Ammonium persulfate	65 µl	65 µl	65 µl	65 µl
TEMED	5 µl	5 µl	5 µl	5 µl

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