EFFECTS OF PRENATAL METHAMPHETAMINE EXPOSURE ON LEARNING AND MEMORY AND SYNAPTOGENESIS IN MOUSE HIPPOCAMPUS

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ผลจากการได้รับเมทแอมเฟทามีนขณะอยู่ในครรภ์ต่อการเรียนรู้และความจำและการสร้างไซแนปส์ในสมองส่วนฮิปโปแคมปัสของหนูเมาส์

น.ส.หัฏฐญา เบญญาอภิกุล

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หัฏฐญา เบญญาอภิกุล : ผลจากการได้รับเมทแอมเฟทามีนขณะอยู่ในครรภ์ต่อการเรียนรู้และความจำและการสร้างไซแนปส์ในสมองส่วนฮิปโปแคมปัสของหนูเมาส์. (EFFECTS OF PRENATAL METHAMPHETAMINE EXPOSURE ON LEARNING AND MEMORY AND SYNAPTOGENESIS IN MOUSE HIPPOCAMPUS) อ.ที่ปรึกษาหลัก : ผศ. ภญ. ดร.รัชนี รอดศิริ, อ.ที่ปรึกษาร่วม : รศ. ภญ. ดร.วริษา พงศ์เรขนานนท์

กระบวนการที่เปลี่ยนแปลงอย่างต่อเนื่องในขั้นตอนการพัฒนาสมองระหว่างตั้งครรภ์และแรกเกิดมีความเปราะบางต่อสารพิษต่อระบบประสาทซึ่งก่อให้เกิดความเปลี่ยนแปลงของโครงสร้างและการทำงานของสมอง ฮิปโปแคมปัสมีบทบาทสำคัญในการเรียนรู้และความจำ การสร้างฮิปโปแคมปัสได้รับอิทธิพลจากการใช้สารเสพติดในช่วงการพัฒนาสมองเช่นในขั้นการเจริญพัฒนาเซลล์ประสาทและการสร้างไซแนปส์ Brain-derived neurotrophic factor (BDNF) ควบคุมการพัฒนาเซลล์ประสาทและ neuroplasticity ผ่านตัวรับ TrkB การใช้เมทแอมเฟทามีนและความเสี่ยงในการได้รับเมทแอมเฟทามีนขณะตั้งครรภ์เพิ่มขึ้นในปัจจุบัน งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาผลของการได้รับเมทแอมเฟทามีนขณะอยู่ในครรภ์ต่อการเรียนรู้และความจำและศึกษาผลต่อลักษณะทางสัณฐานวิทยาของเซลล์ประสาทและการสร้างไซแนปส์ รวมถึงกลไกการเกิดพิษต่อระบบประสาทที่เกี่ยวข้องกับโปรตีนในวิถีสัญญาณ BDNF-TrkB หนูเม้าส์สายพันธุ์ C57BL/6JNc ที่ตั้งครรภ์จะได้รับเมทแอมเฟทามีนขนาด 5 มิลลิกรัมต่อกิโลกรัมหรือน้ำเกลือ ฉีดใต้ผิวหนังอายุครรภ์วันที่ 8 ถึง 15 เซลล์ประสาทเพาะเลี้ยงเตรียมจากฮิปโปแคมปัสของตัวอ่อนอายุครรภ์ 16.5 วัน เซลล์เพาะเลี้ยงฮิปโปแคมปัสใช้สำหรับศึกษาสัณฐานวิทยาของเดนไดรต์และแอกซอน การสร้างไซแนปส์และการแสดงออกของโปรตีนในวิถีสัญญาณ BDNF-TrkB สำหรับการศึกษาแบบ in vivo ในลูกหนูวัยรุ่น (อายุ 6 สัปดาห์) และวัยผู้ใหญ่ (อายุ 12 สัปดาห์) ทำการศึกษาพฤติกรรมใน open-field test, novel object recognition (NOR), novel location test (NLT), elevated plus maze (EPM) and forced swimming test (FST) สมองของลูกหนูอายุ 14 วัน หนูวัยรุ่น และหนูวัยผู้ใหญ่ถูกเก็บมาศึกษาการแสดงออกของโปรตีนบริเวณ presynapse (synapsin-I), postsynapse (PSD-95) และโปรตีนในวิถีสัญญาณ BDNF-TrkB การได้รับเมทแอมเฟทามีนขณะอยู่ในครรภ์ทำให้หนูวัยรุ่นเพศเมียมีความจำบกพร่องในการทดลอง NLT ในขณะที่หนูวัยรุ่นเพศผู้ที่ได้รับเมทแอมเฟทามีนขณะอยู่ในครรภ์มีความจำบกพร่องในการทดลอง NOR นอกจากนี้การได้รับเมทแอมเฟทามีนขณะอยู่ในครรภ์ทำให้หนูวัยผู้ใหญ่ทั้งเพศเมียและเพศผู้มีความจำบกพร่องทั้งในการทดสอบ NOR และ NLT อย่างไรก็ตามไม่มีผลต่อพฤติกรรมการเคลื่อนที่และระดับความวิตกกังวลและซึมเศร้า ในเซลล์ประสาทฮิปโปแคมปัสเพาะเลี้ยงจากหนูที่ได้รับเมทแอมเฟทามีนมีจำนวนแขนงเดนไดรต์ลดลงและความยาวและเส้นผ่านศูนย์กลางของแอกซอนเล็กลง จำนวนของ presynapse ลดลงและพื้นที่ร่วมกันระหว่างไซแนปส์เล็กลงในกลุ่มที่ได้รับเมทแอมเฟทามีน การแสดงออกของโปรตีนบริเวณ presynapse ลดลงในหนูอายุ 14 วันที่ได้รับเมทแอมเฟทามีนขณะอยู่ในครรภ์ แต่หนูวัยรุ่นและวัยผู้ใหญ่มีการแสดงออกของโปรตีนบริเวณ postsynapse เพิ่มขึ้น การได้รับเมทแอมเฟทามีนขณะอยู่ในครรภ์ลดการแสดงออกของ TrkB ในเซลล์ประสาทฮิปโปแคมปัสเพาะเลี้ยงแต่ระดับการแสดงออกของโปรตีนในวิถีสัญญาณ BDNF-TrkB ในเนื้อเยื่อฮิปโปแคมปัสไม่เปลี่ยนแปลง การแสดงออกของโปรตีน BDNF เพิ่มขึ้นใน prefrontal cortex และ striatum ของหนูอายุ 14 วันที่ได้รับเมทแอมเฟทามีน การศึกษานี้แสดงผลระยะยาวของการได้รับเมทแอมเฟทามีนขณะอยู่ในครรภ์ต่อการเกิดการเรียนรู้และความจำบกพร่องในหนูวัยรุ่นและวัยผู้ใหญ่ เป็นผลจากการรบกวนการเจริญเติบโตของเซลล์ประสาทในฮิปโปแคมปัสและการสร้างไซแนปส์ในสมองที่กำลังพัฒนา การเปลี่ยนแปลงของโปรตีนในวิถีสัญญาณ BDNF-TrkB และโปรตีนบริเวณ presynapse ในระยะต้นของชีวิตส่งผลต่อการปรับตัวของโปรตีนบริเวณ postsynapse

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Hattaya Benya-aphikul : EFFECTS OF PRENATAL METHAMPHETAMINE EXPOSURE ON LEARNING AND MEMORY AND SYNAPTOGENESIS IN MOUSE HIPPOCAMPUS. Advisor: Asst. Prof. RATCHANEE RODSIRI, Ph.D. Co-advisor: Assoc. Prof. VARISA PONGRAKHANANON, Ph.D.

The dynamic processes of brain development during perinatal period are vulnerable to neurotoxic insults leading to changes in brain structure and functions. Hippocampus plays an important role in learning and memory. Hippocampal formation can be influenced by substance used during brain developmental processes including neuron differentiation and synaptogenesis. Brain-derived neurotrophic factor (BDNF) regulates neuron development and neuroplasticity via its receptor TrkB. Methamphetamine (MA) abused and the risk of MA exposed during pregnancy have increased recently. The present study aimed to elucidate the effects of prenatal MA exposure on learning and memory. Prenatal effects of MA on neuronal morphology and synaptogenesis, and the underlying neurotoxic mechanism involving BDNF-TrkB signaling pathway were also determined. Pregnant C57BL/6JNc mice were treated with either MA (5 mg/kg, s.c.) or normal saline on gestation day (GD) 8-15. Primary hippocampal cultures were prepared from embryos at GD16.5. Hippocampal neurons were used to examine morphology of axons and dendrites, synaptogenesis and BDNF-TrkB signaling protein expression. For *in vivo* study, adolescent (6 weeks old) and adult mice (12 weeks old) were conducted behavioral studies in open-field test, novel object recognition (NOR), novel location test (NLT), elevated plus maze (EPM) and forced swimming test (FST). Brains from prenatal day 14 (PND14), adolescent and adult mice were collected to determine presynaptic (synapsin-I), post-synaptic (PSD-95), and BDNF-TrkB signaling protein expression. Prenatal MA exposure impaired NLT performance in female adolescent mice while MA-exposed male adolescent mice showed memory deficit in NOR test. Moreover, prenatal MA exposure caused memory impairments in NOR and NLT in female and male adult mice. However, there were no effects on locomotor activity, anxiety and depression levels. MA-exposed hippocampal primary culture demonstrated decreases in secondary dendritic branches, axonal length and diameter. The number of presynaptic terminals and co-localization between pre- and postsynapse in MA group were also declined. The expression levels of presynaptic protein were decreased in the hippocampus of MA-exposed PND14 mice, but an increase in postsynaptic protein expression levels were presented in adolescent and adult mice. Prenatal MA exposure reduced TrkB expressions in primary cultures, while the expression levels of BDNF-TrkB signaling proteins in hippocampal tissue were not changed. The increased BDNF expression was observed in the prefrontal cortex and striatum of MA-exposed PND14 mice. This study showed the long-lasting effects of prenatal MA exposure on learning and memory impairments in adolescent and adult mice. These effects resulted from the disruptions of hippocampal neuronal growth and synaptogenesis in the developing brain. Alterations of BDNF-TrkB signaling and presynaptic proteins were found in early age, which lead to an adaptation in postsynaptic proteins.

Field of Study:	Pharmacology and Toxicology	Student's Signature .....
Academic Year:	2020	Advisor's Signature .....
		Co-advisor's Signature .....

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Hattaya Benya-aphikul

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

BDNF	=	brain-derived neurotrophic factor
BSA	=	bovine serum albumin
CAM	=	cell adhesion molecules
CaMKII	=	Ca^{2+} /calmodulin-dependent protein kinase II
CRE	=	Ca^{2+} response element
CREB	=	cAMP-responsive element binding protein
DA	=	dopamine
DAT	=	dopamine transporters
DG	=	dentate gyrus
DIV	=	days in vitro
DMEM	=	Dulbecco's Modified Eagle Medium
DTI	=	diffusion tensor imaging
ECL	=	electrochemiluminescence
ED	=	embryonic day
EGTA	=	ethylene glycol tetraacetic acid
EPM	=	elevated plus maze
ER	=	endoplasmic reticulum
FA	=	fractional anisotropy
Fig	=	figure

FST	=	forced swimming test
G	=	gram
GD	=	gestation day
IACUC	=	Institutional Animal Care and Use Committee
IgG	=	immunoglobulin G
LTP	=	long-term potentiation
MA	=	methamphetamine
MAO	=	monoamine oxidase
MAP2	=	microtubule-associated protein2
mg	=	milligram
mm	=	millimeter
mM	=	millimolar
NGF	=	nerve growth factor
NLT	=	novel location test
NMDAR	=	N-methyl-D-aspartate receptor
NOR	=	novel object recognition
NPCs	=	neural progenitor cells
NSS	=	normal saline solution
NT3	=	neurotrophin3
NT4	=	neurotrophin4
p-CREB	=	phospho-cAMP-responsive element binding protein

p-TrkB	=	phospho-tropomyosin-related kinase
$p75^{NTR}$	=	p75 neurotrophin receptor
PBS	=	phosphate-buffered saline
PET	=	positron emission tomography
PFA	=	paraformaldehyde
PND	=	postnatal day
PSD	=	postsynaptic density
S	=	second
S.C.	=	subcutaneous
SDS	=	sodium dodecyl sulfate
SDS-PAGE	=	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBS	=	tris-buffered saline
TrkB	=	tropomyosin-related kinase
USF	=	upstream stimulatory factor
VMAT2	=	vesicular monoamine transporter-2

CHAPTER I

INTRODUCTION

1.1 Background and rationale

The developmental processes of nervous system consist of many dynamic events such as proliferation, migration, differentiation and synaptogenesis. These events cover from the early embryonic stage to complete in the postnatal period (Spreen et al 1995). Developing brain was more vulnerable to extrinsic stimuli than adulthood due to its immature and instability of structure and molecular substances (Semple et al 2013). Previous study demonstrated a long-lasting effect of prenatal drug exposure on aberrant behaviors or psychiatric disorders in children and adults (Guille & Aujla 2019).

Most brain activities during developmental stage are regulated by the neurotrophin family protein especially brain-derived neurotrophic factor (BDNF). BDNF signaling via TrkB receptor modulates neurite growth and synaptogenesis (Sonoyama et al 2020). Upstream and downstream of BDNF cascades also associate with cAMP-responsive element binding protein (CREB) transcription factor (Autry & Monteggia 2012). In mature brain, BDNF plays a pivotal role in regulation of brain plasticity in learning and memory process. An activation of CREB promotes neuronal growth, brain plasticity and memory consolidation , which functions in an activity-dependent manner (Amidfar et al 2020). Thus, the deviate expression of BDNF-TrkB and CREB can cause learning and memory impairments.

Methamphetamine (MA) has been widely used as substance abuse throughout the world especially in Thailand. Drug abuse in women of childbearing age has increased greatly (Kanato et al 2020). MA readily crosses placental barrier and distributes to all organs of fetus. MA and its active metabolite, amphetamine, are detected in the fetal brain following maternal injection in animals (Burchfield et al 1991). In the brain, MA affects the monoamine neurotransmitter system by inhibiting reuptake transporters and stimulating neurotransmitter release (Panenka et al 2013). Neurotoxicity of MA results from an excitotoxicity and oxidative stress, which are generated by excess dopamine auto-oxidation. Previous studies of prenatal MA

exposure reported the negative effects on brain development and ongoing cognitive deficit in animals and humans (Kwiatkowski et al 2018, Petrikova-Hrebickova et al 2021, Warton et al 2018).

Hippocampus is the important brain areas that accounts for learning and memory. Hippocampal formation can be influenced by many stimuli including drug use during prenatal neurogenesis, differentiation and synaptogenesis (Bernaskova et al 2017, de Salas-Quiroga et al 2020). Synaptic transmission in the hippocampus is mainly responsible in learning and memory processes. Therefore, alterations of neuronal morphology and synaptogenesis can lead to memory impairment (Sağır 2021).

This study aimed to determine the effect of prenatal MA exposure on neurobehaviors particularly learning and memory function. Here, by primary hippocampal cultures from MA-treated embryos, neurite growth and synaptic formation of developing brain were investigated whether the alterations might contribute to memory impairment. The expression levels of BDNF-related proteins were examined to determine the underlying neurotoxic mechanisms of MA.

1.2 Research questions

1. Does prenatal MA exposure produce a long-term effect on learning and memory?
2. Does prenatal MA exposure affect synaptogenesis in developing brain and adolescent and adult mice?
3. What are the toxic mechanisms of MA in developing brain and adolescent and adult mice?

1.3 Objectives

1. To investigate the effect of prenatal MA exposure on learning and memory in each age and gender of the offspring.
2. To examine the effect of prenatal MA exposure on neuronal morphology and synaptogenesis.

3. To elucidate the underlying toxic mechanisms of MA involving BDNF-TrkB signaling pathway.

1.4 Hypothesis

1. Prenatal MA exposure can impair learning and memory function in adolescent and adult mice.
2. Prenatal MA exposure can affect neuronal growth and synaptogenesis which operate via the BDNF-TrkB signaling pathway.

1.5 Conceptual framework

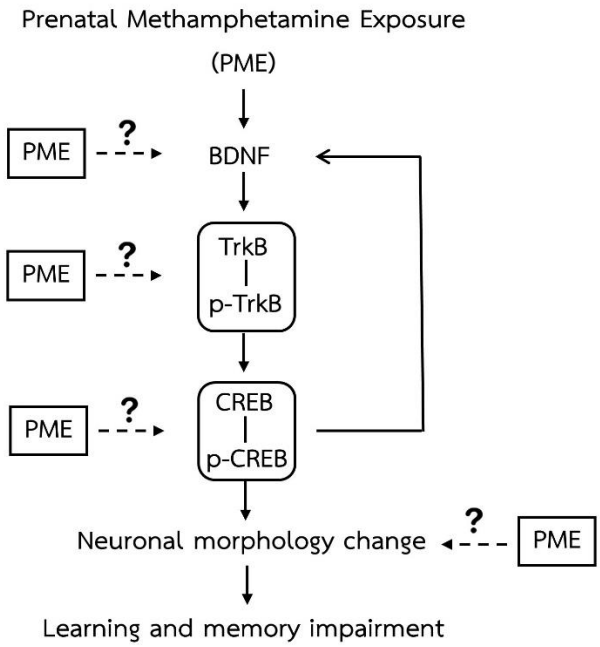

Figure 1 Conceptual framework

1.6 Expected benefits

This study will provide an insight into the neurotoxicity and neurotoxic mechanisms of prenatal MA exposure on synaptogenesis which account for learning and memory deficit. Knowledge about negative impact on learning and memory will raise the awareness of children's problems related to drug abuse during pregnancy.

CHAPTER II

Literature review

2.1 Brain development

2.1.2 Development of the nervous system

Neural development comprises of many dynamic processes to become a functional neuron at specific brain area. After a completion of neurulation, neuroblast, a nerve cell precursor, enters the proliferative phase in a specific layer called ventricular zone, which starts around gestation day 9.5 (GD9.5) in rodents (Vitalis & Verney 2017). Post-mitotic cells have to migrate and aggregate to form their final lamination. The timeline of these processes can vary from area to area of the brain. There are many aspects of neuronal differentiation from cell body development to axonal, dendritic and synaptic formation. Neurite growth deals with an element of microtubules and microfilaments which mainly controlled via cell adhesion molecules (CAM). Cell bodies reach their final place and start to elongate and polarize to change into asymmetry; thus, they appoint which neurite will become an axon (Ackerman 1992). Myelination and synaptogenesis initiates in late prenatal and continues through the early postnatal period (Babikian et al 2010). Although blood-brain barrier has been generated since embryogenesis, the defending mechanism of developing brain is not similar to adulthood. Most sorts of injury during brain development are indicated as age-dependent vulnerability, especially developing brain that exhibits a distinguished susceptibility to oxidative stress and apoptosis (Semple et al 2013).

Axon and dendrite are characterized with their specific markers within first week of the primary hippocampal cultures prepared from rat brains (Dotti et al 1988). The sequence of neuronal outgrowth *in vitro* is parallel to those *in vivo.* There are 5 stages of neuronal outgrowth in dissociated cells, which start from immature neurite protrusion in stage 1 to the terminal differentiation in stage 5. Pyramidal neurons in stage 5 are characterized by a mature axon, dendritic branching and functional synapse. The terminally differentiated pyramidal neurons *in vivo* are defined with

same markers in stage 5, which is achieved in the first postnatal week of infant (Polleux & Snider 2010).

2.1.2 Neurite growth pathway

Axon and dendrite are originated from the polarization step during neuronal development. There are many factors modulated the initiation and outgrowth of neurite in both cellular and molecular level (Polleux & Snider 2010). Neuronal polarity is essentially regulated by the selective degradation of protein kinase AKT (Yan et al 2006). The remaining axon is spatially enriched with the active form of AKT and GTPase Rap1b (Schwamborn et al 2007). After axon determination, the local protein translation is required to maintain and stimulate an outgrowth of developing axon and synaptic plasticity (Job & Eberwine 2001). Previous studies revealed an important role of the neurotrophin family in neurite growth. They function as an extracellular cue to enhance the local protein synthesis in both dendrites and axons (Hengst et al 2009, Kang & Schuman 1996). Nerve growth factor (NGF) and netrin-1 triggers the translation of Par3 mRNA in developing axons (Kang & Schuman 1996). In term of microtubule dynamics in axon, a microtubule plus-end binding protein (APC) plays a pivotal role to stabilize microtubule. This pathway is controlled by a phosphorylation by GSK-3β via PI3K signal (Shi et al 2004). Interestingly, GSK-3 can also phosphorylate MAP1b and Tau proteins to reduce their abilities (Gonzalez-Billault et al 2004).

2.2 Brain-derived neurotrophic factor

The neurotrophin family composes of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin3 (NT3) and neurotrophin4 (NT4). Each of them binds to an individual receptor and possesses different functions. NGF promotes neural survival and differentiation while BDNF is responsible for neurite outgrowth in neuronal precursors (Ahmed et al 1995). BDNF involves in many synaptic and neuronal processes including changes in spine morphology, increases in N-methyl-D-aspartate receptors (NMDARs) conductance and synaptic plasticity (Alonso et al 2004, Lin et al 1998). BDNF specifically binds to tropomyosin-related

kinase (TrkB) and $p75^{NTR}$ receptor resulting in an auto-phosphorylation of these tyrosine kinase receptors (Hengst et al 2009). Phosphorylated TrkB receptor induces several signaling pathway as shown in Figure 2 (modified from (Autry & Monteggia 2012)). Furthermore, BDNF can modulate axonal growth and differentiation via PKA-dependent LKB1 phosphorylation (Shelly et al 2007). In mature brain, BDNF conducts synaptic plasticity in activity-dependent manner (Waterhouse & Xu 2009). BDNF localizes in both pre- and postsynaptic areas. Presynaptic BDNF signaling stimulates neurotransmitter release, while postsynaptic BDNF partially strengthen ion channel function. The BDNF-TrkB signaling pathway also induces local protein synthesis which is necessary for synaptic plasticity and memory formation. A reduction of BDNF in animal models worsen hippocampal long-term potentiation (Kang & Schuman 1996, Lu et al 2008).

The adenosine 3',5'-monophosphate (cAMP) response element-binding protein (CREB) is a transcription factor, which is associated with many neuronal response processes (Sheng et al 1991). The downstream of BDNF-TrkB pathway activates many gene expressions via CREB. On the other hand, CREB manipulates BDNF expression together with upstream stimulatory factor (USF) 1/USF2 (Tabuchi et al 2002). Calcium influx evokes a phosphorylation of CREB which then binds to Ca^{2+} response element (CRE) site on *bdnf* gene, so BDNF transcription is elevated (Tao et al 1998).

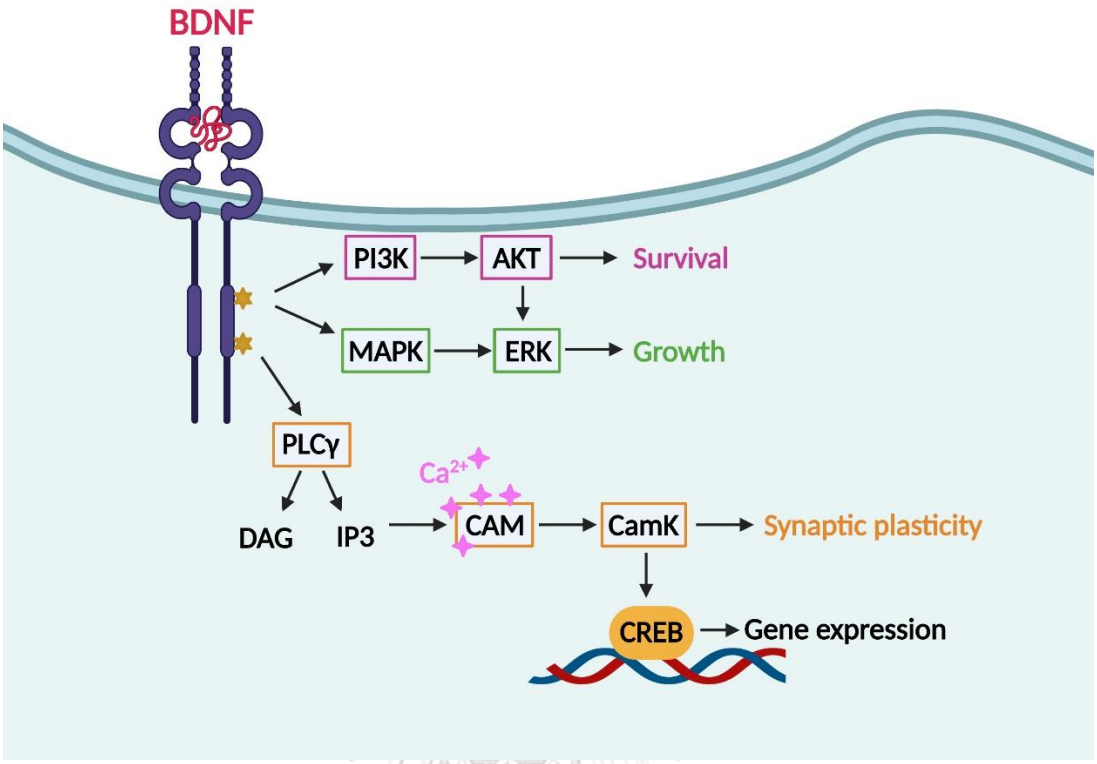

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Figure 2 BDNF-TrkB pathway regulating many neuronal activities

2.3 Synaptic plasticity and learning and memory

2.3.1 Synaptogenesis

Synapses are necessary structure that accomplish the communications in neural circuit through chemical and electrical transmission. An initiation of synapse formation begins after neuronal differentiation stage in developing embryo's brains. An ongoing development throughout perinatal processes expands density of synapse and strengthens functions (Thion & Garel 2017). Early steps of synaptogenesis include postsynaptic protrusion from dendritic spines and extension of presynaptic growth cones. The extending neurites are guided to innervate to its targets, then making a physical contact between pre- and postsynaptic terminals (Munno & Syed 2003). A

competence to maintain and eliminate synapse subsequently proceeds overtime. Since the density of synapse relies on axonal and dendritic branching, BDNF indeed modulates and stabilizes the synapse formation (Hu et al 2005). A deletion of TrkB receptor induces decreases in presynaptic boutons and defect of synaptic efficacy with less protein-related neurotransmitter exocytosis (Martínez et al 1998). For postsynaptic feature, BDNF expressed the effect on enlargement and pruning of dendritic spines of hippocampal neurons (An et al 2008). Furthermore, BDNF can increase dendritic spines of hippocampal CA1 pyramidal neurons via ERK-signaling pathway (Alonso et al 2004). The cAMP second messenger facilitates BDNF-induced TrkB phosphorylation, and the trafficking of TrkB to postsynaptic density (PSD) zone in dendritic spine (Ji et al 2005). Ras-PI3K-Akt-mTOR is another downstream pathway of BDNF which regulates dendritic spine and arbor shape in CA1/ CA3 neurons of hippocampal cultures (Kumar et al 2005). However, some experiments show that the effect of BDNF on spine density varies in each brain area. BDNF knockout mice are observed with more sensitive in decline in dendritic spines in the striatum than in the hippocampus (Rauskolb et al 2010).

2.3.2 Synaptic plasticity involved in learning and memory

Brain has been remodeled structurally and functionally throughout the lifespan, including neural circuits and signal transmissions. This property is called plasticity that associates with many brain functions. Synapse is also remarkably plastic because its ability to modify the strength of firing. There are many types of synaptic plasticity, but long-term potentiation plays the most crucial role in learning and memory. Learning elicits an experience-dependent synaptic plasticity of hippocampal neurons. Memory formation and storage are produced by a synchronously activation of two-connected neurons. An increase in the strength of these synaptic connections is needed to preserve memory. This phenomenon is called long-term potentiation (LTP) which occurs when brain is stimulated by a constant high-frequency, then increases in synaptic transmission (Brady et al 2011).

LTP possesses 2 phases; early and late phase, which express through different molecular mechanisms. The early phase of LTP lasts for 1 to 3 hours, and operates

with Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) as a signaling molecule. The activated CaMKII by intracellular Ca^{2+} results in a translocation of **α**-CaMKII to the PSD zone and then potentiates synaptic transmission. On the other hand, late phase LTP extends longer potentiation and elicits slower. Due to this late status, it requires gene transcription and protein synthesis especially through CREB and BDNF pathway (Kandel et al 2015). A deletion of BDNF in CA1 and CA3 of mouse hippocampus confirmed that BDNF in presynaptic area contributes to LTP induction, while BDNF in postsynaptic area facilitates LTP maintenance. A role of BDNF in LTP also conducts via pre- and postsynaptic TrkB receptors (Lin et al 2018).

2.4 Hippocampus

The hippocampus is the major compartment of limbic system and necessary for learning and memory function. It is responsible for explicit or declarative memory storage, which is a conscious recall of information in long-term memory. In learning processes, the hippocampus also plays a pivotal role in spatial-associated learning (Schacter DL 2015).

The hippocampal formation composes of 3 main sub-regions; dentate gyrus (DG), CA1 and CA3. Three synaptic pathways are classified in the hippocampus (Fig. 3). First is the perforant pathway where DG receives information from the adjacent entorhinal cortex. Second is the mossy fiber pathway where the information is transferred from DG to CA3 area. The last transmission is the Schaffer collateral pathway where the information from CA3 is transferred to CA1 area before relaying to other involved structures (Schultz & Engelhardt 2014).

The hippocampus constitutes of different cell types which are distinct in organization and developmental stage. Pyramidal cells are the main functional neurons located in CA3 and CA1 areas while granule cells are in DG. The period of neurogenesis in most cortical and subcortical regions covers from GD9.5 to postnatal day (PND) 15. The hippocampal develops in a perinatal stage because it composes of two cell types (Semple et al 2013). The pyramidal cells are generated prenatally, while granule cells mainly develop in the postnatal stage. Hippocampal neurons are highly proliferated during GD14 to GD17 (Rice & Barone Jr 2000). The proliferated

cells migrate and then differentiate to form synapses. Differentiation process is required to define the terminal phenotype of neurons, which can be characterized by shape, size or an expression of operative proteins. Synaptogenesis process is an activity-dependent condition that modulates many brain functions including learning and memory (Reznikov 2012). Thus, these stages of neuronal development are vital for appropriate brain functions in later life.

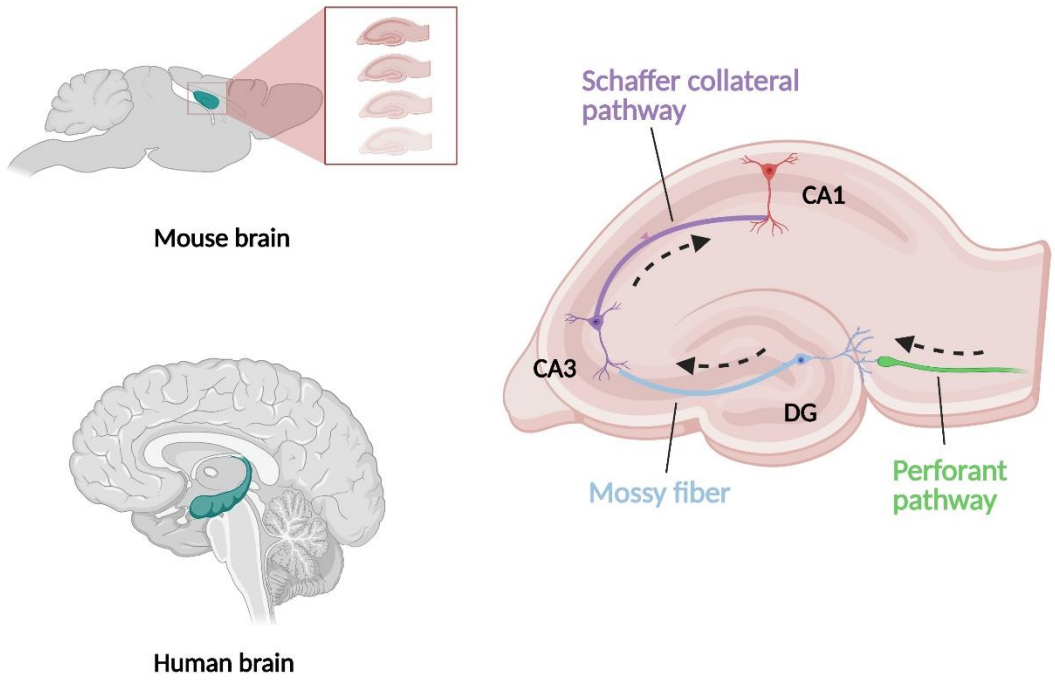

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Figure 3 Synaptic pathways in the hippocampus

2.5 Methamphetamine

MA is a synthetic potent psychostimulant that commonly abused worldwide. This substance is classified as the amphetamine derivative class of psychoactive drugs. MA is synthesized in both pure dextro-methamphetamine and racemic mixture. Pure methamphetamine hydrochloride is normally found in crystalline form, also known as 'ice'. Ice or crystal meth is the most popular drug abuse among those amphetamine derivatives. Drug users often abuse in powders or tablets of MA and

crystalline MA via various routes; such as oral, injection and nasal (snort) (Courtney & Ray 2014). In Thailand, MA is scheduled by law in category I of "Narcotics Act B.E. 2522".

2.5.1 Epidemiology

MA is initially synthesized for medical reasons. Its psychological effects are higher than its progenitor compound ‘amphetamine’. Several years after the first launch, MA was intentionally used for non-medical purposes due to its strong psychostimulant effects. Rates of abuse had been rapidly increased; thus, it was restricted and became illegal. MA was marketed predominantly in the past decades, especially in South-East Asia. The United Nations Office of Drug and Crime revealed that five-year change of global seizures has increased by 158% (UNODC 2016). Nowadays, crystal meth has continued to be prevailing among teenagers. Adolescent females are likely to use MA over other illicit drugs (Kidd et al 2019, Kittirattanapaiboon et al 2017). Therefore, drug use in child-bearing age is considered as one of the major public health issues. MA-abused pregnant women are more likely to have co-morbidity of substance use disorders and psychiatric disorders (Wouldes et al 2013).

2.5.2 Physiochemical properties (National Center for Biotechnology Information 2021)

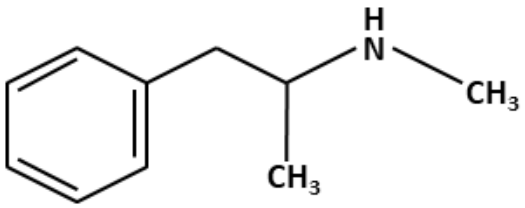


Figure 4 Chemical structure of methamphetamine

Chemical name: Methamphetamine

IUPAC name: (2S)-N-methyl-1-phenylpropan-2-amine

Molecular Formula: $C_{10}H_{15}N$

Molecular weight: 149.237 g/mol

Solubility (in water): 0.5 g/mL

log P: 2.07

pKa: 9.87

Table 1 Solubility of a different form of MA in various organic solvents (National Center for Biotechnology Information 2021)

Form	Base	Hydrochloride
Acetone	Soluble	very slightly soluble
Chloroform	Soluble	Freely soluble
Ether	Soluble	Insoluble
Hexane	Soluble	Insoluble
Methanol	Soluble	Freely soluble
Water	Slightly soluble	Freely soluble

2.5.3 Pharmacokinetic profile

Routes of administration have an impact on pharmacokinetic profile of MA. Intravenous injection, smoke and intranasal administration cause a higher bioavailability than oral administration (Panenka et al 2013). The metabolism pathway of MA is conducted primarily by CYP2D6 to gain the major metabolites; p-hydroxymethamphetamine and amphetamine. Amphetamine is a pharmacologically active metabolite found in many organs, including brain (Won et al 2001b).

Distribution of MA in whole human body was studied by Positron Emission Tomography (PET) scanning. After intravenous injection, MA distributes rapidly to lung and heart, followed by spleen, kidney, pancreas, brain, stomach and liver. Rate of clearance is slowest in the brain, liver and stomach (more than 75 minutes) (Volkow et al 2010). MA distribution was also studied in pregnant animals to evaluate the disposition in fetuses. Several studies showed placental transfer of MA in different

animal species and also in human (Perez et al 2021, Rambousek et al 2014). Pharmacokinetic studies in sheep revealed that MA promptly crosses placenta after intravenous injection, and fetal clearance rate was lower than in the mother leading to the longer half-life of MA in the fetus (Burchfield et al 1991). MA entered the fetal vascular system and distributed to all tissue parts. The highest tissue-plasma ratio of MA in the fetus was found in lung. MA also distributed and accumulated in the fetal brain after 2-hour administration to pregnant ewe (Burchfield et al 1991). When MA (40 mg/kg s.c.) was given to pregnant mice on gestation day 14 (GD14), the concentration of MA in fetal brain was approximately three to four-fold less than that in maternal brain (Won et al 2001a). The time-concentration profile in fetal brain revealed the peak at 1 hour after maternal injection, and MA was still detected at 4 hours after maternal injection. MA concentrations in the different embryo brain areas were varied (Won et al 2001a). In addition, repeated MA administration produced higher level of MA in plasma and brain dialysate than a single dose (Fujimoto et al 2007).

2.5.4 Pharmacology and toxicology

2.5.4.1 Pharmacological properties of MA

Pharmacological effects of MA results from the alterations of monoamine neurotransmitter levels. MA exhibits multiple sites of action at synaptic terminal, which primarily inhibits dopamine transporters (DAT) causing an increase in synaptic dopamine (DA) levels. Plasmalemmal DAT is also less expressed to decrease reuptake function. MA inhibits the vesicular monoamine transporter-2 (VMAT2), which regularly uptake monoamine neurotransmitter into the storage vesicles. Therefore, MA directly induces dopamine release from vesicles and subsequently promotes non-exocytic efflux of dopamine. Moreover, MA enhances tyrosine hydroxylase activity to increase a dopamine synthesis, and inhibits monoamine oxidase (MAO) activity which is a DA degradation enzyme (Panenka et al 2013, Sulzer et al 2005).

Dopaminergic and serotonergic neurons are the target sites of MA neurotoxicity. MA destroys dopaminergic and serotonergic nerve endings resulting in the long-term decreases in dopamine and serotonin levels (Wagner et al 1980). MA

causes dopamine and serotonin depletion together with the reduction of tryptophan hydroxylase and tyrosine hydroxylase activity in many brain areas (Hotchkiss & Gibb 1980). DAT function decreases significantly after chronic self-administration of MA in rats (McFadden et al 2012). Clinical study also reports the loss of DAT density in MA abusers (McCann et al 1998). While MA-induced damage on dopaminergic systems is found in the striatum, MA-induced serotonin deficit is predominantly investigated in the hippocampus. VMAT-2 functions in serotonergic terminals are disrupted leading to persistent serotonin deficit (Rau et al 2006).

There is strong evidence supporting the role of oxidative stress in MA-induced neuronal death. MA increases lipid peroxidation products and hydroxyl radicals *in vivo* (Yamamoto & Zhu 1998). Excessive release of dopamine after MA injection is auto-oxidized to form reactive oxygen species (LaVoie & Hastings 1999). Apart from dopamine, extracellular glutamate level is elevated after repeated MA administration (Nash & Yamamoto 1992). Glutamate is associated with excitotoxicity resulting from redundant calcium influx and superoxide production (Prehn 1998, Schinder et al 1996). Glutamate receptor antagonist can alleviate neuronal damage induced by MA (Sonsalla et al 1989). In addition, the inhibition of electron transport chain in mitochondria is hypothesized as a cause of oxidative stress. MA inhibits mitochondrial complex II-III function in the striatum, but not in the hippocampus (Brown et al 2005).

2.5.4.2 Prenatal effects on neurotoxicity

Methamphetamine abuse during pregnancy causes changes in brain structure of children. A study in MA-exposed children revealed the significant transformation in volumes and cortical thickness (Roos et al 2014). A smaller volume of the putamen and hippocampus is also observed in prenatal MA-exposed adolescent children (Chang et al 2004). Considering age of subjects, pre-adolescent children exhibit higher volume of putamen areas than adolescent age. The findings implicate the repercussion of MA on developmental trajectories and diminished brain plasticity. Microstructural brain maturation can be examined noninvasively in children by diffusion tensor imaging (DTI) method. Lower fractional anisotropy (FA) is observed in children who exposed to MA with or without other concurrent drugs. This abnormal

white matter microstructure implies an irregular coherent fiber and myelination in many areas of the brain. An altered microstructure in those specific areas is associated with poorer motor coordination and cognitive impairment in children and adolescence (Chang et al 2016, Roos et al 2015). However, some studies report higher FA in MA-exposed children (Colby et al 2012). The inconsistency can be explained by an effect of gender, polydrug use or even age of attended children.

Drug abuse negatively affects many aspects of neuronal cell development, including cell proliferation, cell differentiation and apoptosis (Willner et al 2014). MA impairs proliferation and self-renewal capacity of hippocampal neural progenitor cells (Baptista et al 2014, Venkatesan et al 2011). *In vitro* study using embryo stem cell-derived neuronal cells demonstrated the outgrowth reduction concurrent with the declining in microtubule-associated protein2 (MAP2) protein expression after MA treatment (Meamar et al 2012). This finding indicates the impairment of cytoskeleton of neurons as MAP is an important cytoskeletal protein located at somatodendritic compartment (Azmitia et al 1995, Whitaker-Azmitia et al 1995).

A few studies have investigated the effects of prenatal MA exposure on neuronal morphology and synapse development *in vivo*. One *in vivo* prenatal study focuses on PSA-NCAM, a neural cell adhesion molecule involved in neurite outgrowth, cell proliferation, myelination and synapse formation. The reduction of PSA-NCAM in the hippocampus is demonstrated in the infant rats prenatally exposed to MA (Baei et al 2017). There is another study reports smaller axons and myelin areas of the optic nerves in MA-treated rat in utero (Melo et al 2008b). Histological abnormalities of brain including ectopic cell cluster within marginal zone of cortex and hemorrhage are reported in prenatally MA-exposed fetuses (Cui et al 2006).

Many studies have considered the neurotoxicity of MA in the aspect of neurobehavioral pattern. Many clinical studies report alterations of neurobehaviors, increases in physiological stress and CNS stress, an impairment of motor development and cognitive deficits in prenatal MA-exposed children (Diaz et al 2014, Kiblawi et al 2014, LaGasse et al 2011, Smith et al 2015, Smith et al 2008, Wouldes et al 2014). Long-term effect of prenatal MA exposure on learning and memory deficit was shown in rats performing the novel object recognition task (Fialová et al 2015).

However, neither effect on initial learning process nor memory retrieval was observed in the morris water maze model (Schutová et al 2008). MA administration in a different gestational stage causes the varied memory function outcomes. MA injection in early phase (GD1-11) did not establish an effect on learning and memory in offspring, while late phase (GD12-22) exposure impaired spatial learning (Hrebíčková et al 2014). These effects of MA are also sex-specific as learning and memory were not deteriorated in female rats prenatally exposed to MA (Macúchová et al 2013a). Taken together, prenatal MA exposure causes long-term consequences to the learning and memory of the offspring. The mechanisms of MA toxicity on learning and memory following prenatal administration should be further investigated.

CHAPTER III

METHODOLOGY

3.1 Materials

3.1.1 Experimental animals

Adult male and female C57BL/6JNc mice (6 weeks old) were purchased from Nomura Siam International Co., Ltd., Thailand and delivered to Animal Research Unit at Faculty of Pharmaceutical Sciences, Chulalongkorn University. Male and female mice were housed separately until they were allowed to mate. Three male mice were housed in one cage while female mice were housed in a group of five. The housing room condition was controlled at 24±2°C with 40-60% humidity and 12-hour light/ dark cycle in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals 2011). Standard food and water were provided ad libitum in polypropylene cages.

Mouse mating was conducted with the harem system, which 1 male mouse was housed with 2-3 female mice overnight. On the next day, vaginal plug was observed to confirm for insemination and defined as gestation day 0 (GD0). Female mouse with insemination was housed separately with enrichments. This animal care and use protocol was approved by the Institutional Animal Care and Use Committee (IACUC), Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand (No. 1633007, 1833013).

According to ethical animal protocol, all mouse was handled with 3R consideration; replacement, reduction and refinement. The inbred mouse (C57BL/6JNc) was used in this study to reduce experimental variability. The number of mice used to collect primary neurons and behavioral test was minimized for appropriate results.

3.1.2 Chemicals

40% Acrylamide/Bisacrylamine (Bio-rad®, USA)

Alexa Fluor® 647 Phalloidin (Cell signaling technology®, USA)

Ammonium Persulfate (Bio-rad®, USA)

Anti-GAPDH Polyclonal Antibody (Millipore, USA)

Anti-GAPDH Monoclonal Anitibody-HRP conjugated (Cell signaling technology®, USA)

Anti-Microtubule-Associated Protein 2 (MAP2) Polyclonal Antibody (Millipore, USA)

Anti-Post Synaptic Density Protein 95, clone 6G6-1C9 Monoclonal Antibody (Millipore, USA)

Anti-Synapsin I Polyclonal Antibody (Millipore, USA)

Anti-Tau-1 Monoclonal Antibody (Millipore, USA)

B-27 Supplement (Gibco®, USA)

BCA protein assay kits (Thermo Scientific, USA)

BLUeye Prestained Protein Ladder (Bio-helix, Tain)

Boric acid (Emsure®, USA)

Bovine serum albumin (SRL, India)

Bromophenol blue (OmniPur®, USA)

CREB Rabbit Monoclonal Antibody (Cell signaling technology, USA)

Cresyl violet (Sigma-Aldrich, USA)

DAPI (ChemCruz®, USA)

Deoxyribonuclease I from bovine pancreas (Sigma-Aldrich, USA)

di-Sodium hydrogen phosphate (Emsure®, USA)

DL-cysteine hydrochloride (Wako®, Japan)

DMEM high glucose medium (Wako®, Japan)

99.5% Ethanol (Emsure®, USA)

Ethylene Glycol Tetraacetic Acid (EGTA) (TCI, Japan)

Fetal Bovine Serum (Gibco®, USA)

Glucose (Merck, USA)

Glycerol (OmniPur®, USA)

Glycine (OmniPur®, USA)

Goat anti-Rabbit IgG, Peroxidase Conjugated Secondary Antibody (Millipore, USA)

Goat anti-Rabbit IgG (H+ L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (ThermoFisher Scientific, USA)

Goat anti-Mouse IgG (H+ L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 568 (ThermoFisher Scientific, USA)

HEPES (Vetec™, USA)

Horse Serum (Gibco®, USA)

L-Glutamine (Gibco®, USA)

Magnesium Chloride (Ajax Finechem, Australia) 77

2-Mercaptoethanol (TCI, Japan)

Methamphetamine (FDA, Thailand)

Neurobasal medium (Gibco® USA)

65% Nitric acid (Emsure®, USA)

Non-fat dried milk (Cell signaling technology, USA)

Normal saline solution (NSS) (Thaiotsuka, Thailand)

NP-40 (Applichem, USA)

Papain (Nacalai®, Japan)

Paraformaldehyde (Sigma-Aldrich, USA)

Penicillin Streptomycin (Gibco®, USA)

Phenylmethylsulfonyl Fluoride (Sigma-Aldrich, USA)

Phospho-CREB (Ser133) Rabbit Monoclonal Antibody (Cell signaling technology, USA)

Poly-D-lysine hydrobromide (Sigma-Aldrich, USA)

Potassium Chloride (Emsure®, USA)

Protease inhibitor (Roche, USA)

Sodium Chloride (Wako®, Japan)

Sodium Dodecyl Sulfate (OmniPur®, USA)

Sodium Fluoride (Emsure®, USA)

Sodium orthovanadate (Sigma-Aldrich, USA)

Sodium Pyruvate (Gibco®, USA)

SuperSignal® West Pico chemiluminescent substrate (Thermo Scientific, USA)

Tris (hydroxymethyl) aminomethane (OmniPur®, USA)

Triton X (Sigma-Aldrich, USA)

TRIzol® Reagent (Sigma-Aldrich, USA)

Tween-20 (OmniPur®, USA)

Tetramethylethylenediamine (OmniPur®, USA)

3.1.3 Equipment

Animal experiment

Hypodermic needle size 27G x 0.5 inches (Nipro, Corp. Ltd., Thailand)

Insulin syringe, 1 mL (Nipro, Corp. Ltd., Thailand)

Surgical instrument (FST GmbH, Germany)

Cell culture and sample preparation

Microcentrifuge tubes

Microlitre centrifuge

CGG842 laboratory pH meter (SCHOTT Instruments GmbH, Germany)

Homogenizer

Dounce homogenizer (Glas-Col, USA)

Cryostat microtome (LEICA, Germany)

Vortex mixture, model VM300 (Axiom, Germany)

VideoMOT2 system (TSE Systems GmbH, Bad Homburg, Germany)

CO_2 incubator, model HERAcell 150i (Thermo Scientific, MA, USA)

Dry bath Incubator, model EL-02 Dual Block (Major Science Co., Ltd., CA, USA)

3.2 Methodology

3.2.1 Chemical preparation

Methamphetamine Hydrochloride (98.22%) was obtained from Food and Drug Administration, Ministry of Public Health, Thailand. Methamphetamine (MA) was dissolved in 0.9% saline solution (NSS) at the concentration of 0.5 mg/mL. The dose of 5 mg/kg of MA was injected to pregnant mice in this study because this dose was reported with a comparable fetal brain drug concentration to humans (Acuff-Smith et al 1996b). Furthermore, this dose could induce behavior changes with no abortions in pregnant rats (Šlamberová et al 2005).

3.2.2 Experimental design

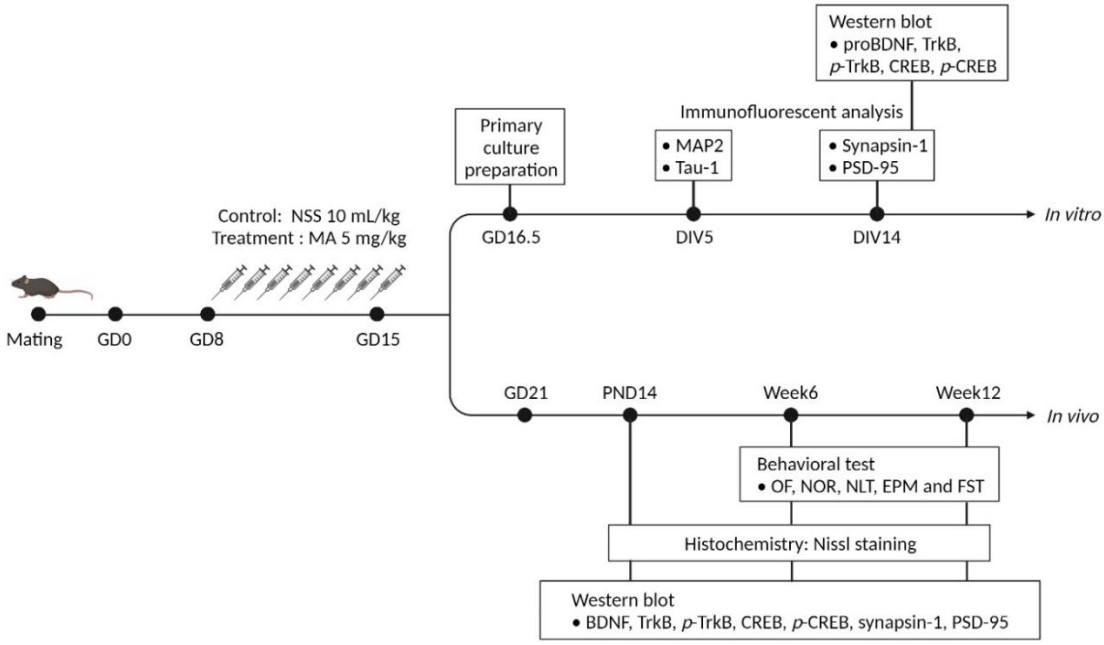


Figure 5 Experimental design

3.2.3 *In vivo* experimental design

This was a randomized design trial. After overnight mating, female mice with vaginal plug were isolated as pregnant mice. These pregnant mice were randomly divided into 2 groups which are control and MA-treated group.

Mice in the control group received NSS 10 ml/ kg by subcutaneous injection for 8 consecutive days (GD8 to GD15) . Mice in MA-treated group received methamphetamine 5 mg/ kg by subcutaneous injection for 8 consecutive days. Pregnant mice were expected to deliver approximately at GD21. One pup from each age (14-day-old, 6- and 12-week-old) and gender from same litter was randomly allocated to experimental groups. The remaining were terminated. Animal experiments were conducted in 3 different ages: PND14 (infant) , 6 weeks old (adolescence) and 12 weeks old (adult). On PND14, one mouse from each litter was euthanized by CO_2 and dissected for brain collection. After weaning on PND21, remaining pups were housed separately by gender until further behavior studies. At

week 6 (PND36-42) and week 12 (PND78-84), one female and one male mouse were tested in these behavioral models: open-field test, novel object recognition (NOR), novel location test (NLT), elevated plus maze (EPM) and forced swimming test (FST). Mice were euthanized by CO_2 after the final test, and whole brain was collected. Brains were separated into left and right hemispheres. The left hemisphere of the brain was kept in 4% paraformaldehyde and sliced in coronal plane using cryostat microtome to evaluate neuronal viability by cresyl violet staining. Tissue from the prefrontal cortex, hippocampus and striatum was isolated from the right hemisphere of the brain and kept in cryovial tube for liquid nitrogen snap freezing, then in -80°c until western blot analysis. A schematic diagram of *in vivo* study is shown in Figure 6.

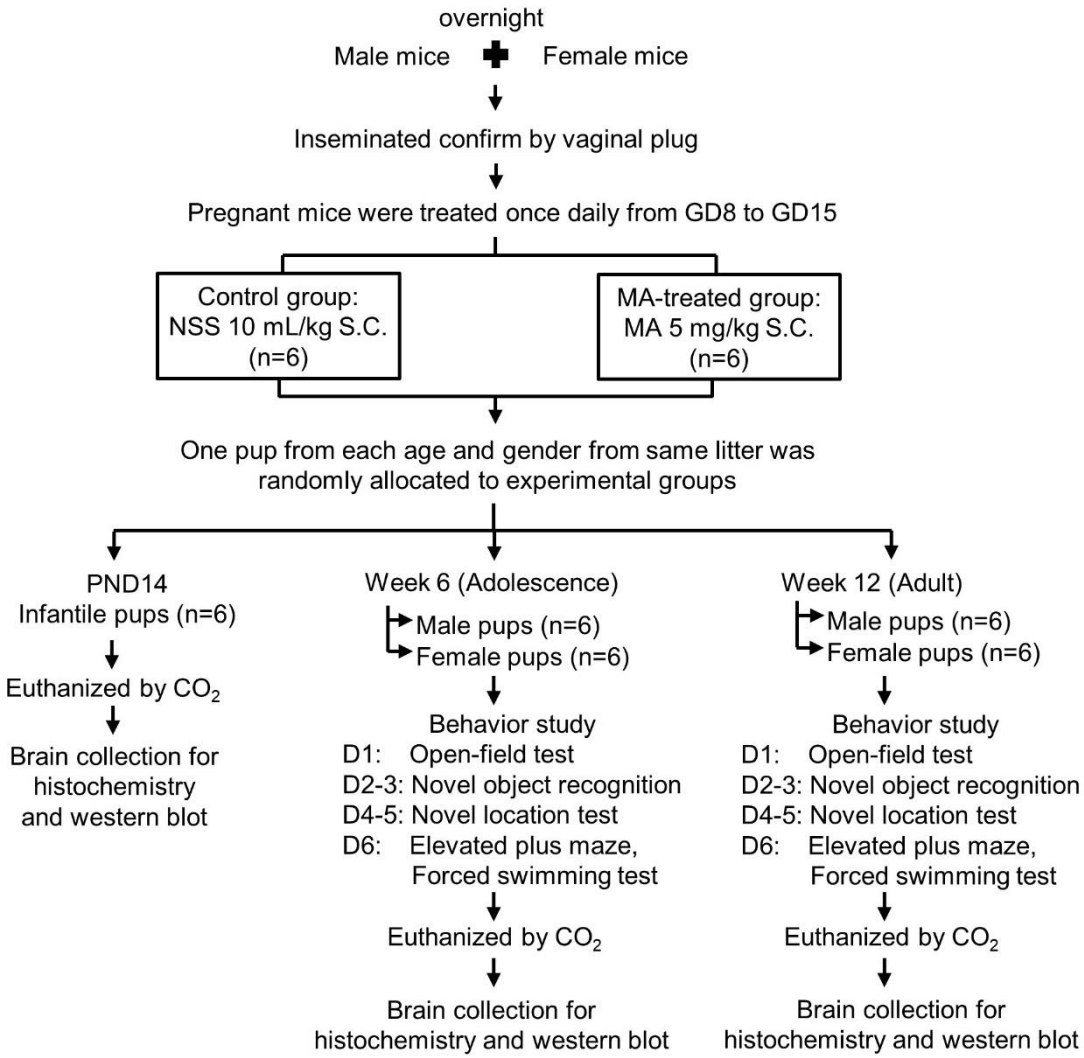


Figure 6 Schematic diagram of animal experiment protocol

3.2.4 Behavioral models

1) Open-field test

Open-field test was used to evaluate the effect of prenatal MA exposure on locomotor activity. Mice were allowed to walk freely in the white box (50 x 50 x 40 cm.) for 5 minutes. The video tracking apparatus was placed over the box to monitor the locomotor activity of mice. Parameters were analyzed as total distance and locomotion time.

2) Novel object recognition (Antunes & Biala 2012)

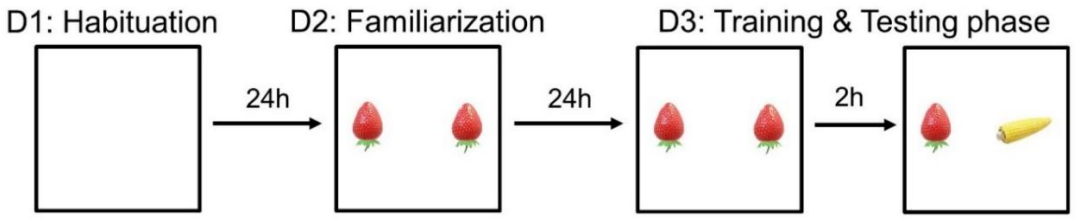


Figure 7 A protocol of novel object recognition apparatus

Learning and memory performance was evaluated by two behavioral models; novel object recognition (NOR) and novel location test (NLT), which used to determine working memory and spatial memory, respectively. NOR was divided into 3 phases: habituation, familiarization and test phases and conducted in the following protocol (Fig. 7).

Day 1, habituation phase: Mice were individually habituated in an empty lid-open arena (25 x 35 x 15 cm.) for 15 minutes.

Day 2, familiarization phase: Mice were trained in an arena with 2 identical objects for 10 minutes. Red strawberry-shaped object was used in this study.

Day 3, test phase: On this day, mice were trained with 2 identical objects again for 10 minutes. Then, mice were removed from an arena and rested in a cage for 2 hours. After an inter-trial interval time, mice were brought back to an arena in which one object was changed to a novel object. Yellow corn-shaped object was used as the novel object in this study. Mice were allowed to explore objects for 5 minutes. The

time spent on object, which was defined by the time when head or nose of mice approaching to objects, was measured as the exploration time. Parameter of the NOR was presented as the discrimination index, which was calculated by (time exploring the novel object – time exploring the familiar object)/(time exploring the novel object + time exploring the familiar object). The preference index was also reported as percent time spent exploring each familiar or novel object, which was calculated by (time spent on each object/total time spent on 2 objects) x 100.

3) Novel location test (Vogel-Ciernia & Wood 2014)

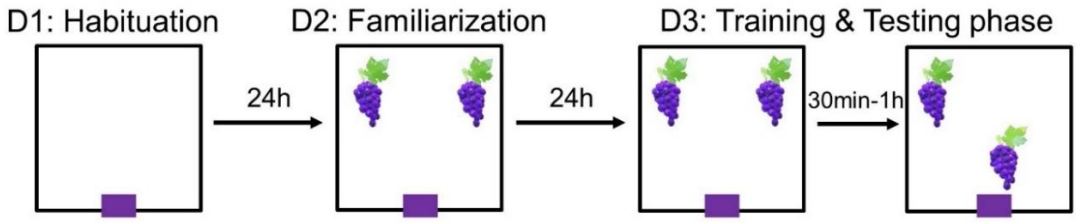


Figure 8 A protocol of novel location test apparatus

The NLT was specifically used for spatial memory determination which profoundly depends on the hippocampal ability. The arena used in this study has a symbol on the wall as a spatial cue. While the novel location test was conducted, one identical object was relocated to a novel location in the arena. This task was also divided into 3 phases: habituation, familiarization and test phases and conducted in the following protocol (Fig. 8).

Day 1, habituation phase: Mice were individually habituated to an empty lid-open arena (25 x 35 x 15 cm.) for 15 minutes. There was a square symbol on one side of arena's wall as a spatial cue.

Day 2, familiarization phase: Mice were trained in an arena with 2 identical objects for 10 minutes. Violet grape-shaped object was used in this study.

Day 3, test phase: On this day, mice were trained with 2 identical objects again for 10 minutes. Then, mice were removed from an arena and rested in a cage for 30 minutes and 1 hour in an experiment for 6-week-old and 12-week-old mice, respectively. After an inter-trial interval time, mice were brought back to an arena in

which one object was relocated to a novel location in the arena. Mice were allowed to explore objects for 5 minutes and time spent at each object was measured. The time spent on object, which was defined by the time when head or nose of mice approaching to objects, was measured as the exploration time. Parameter of the NLT was presented as the discrimination index, which was calculated by (time exploring the object in novel location – time exploring the object in familiar location)/(time exploring the object in novel location + time exploring the object in familiar location). The preference index was also reported as percent time spent exploring each object in familiar or novel location, which was calculated by (time spent on the object in each location/total time spent on the object in two locations) x 100.

3) Elevated plus maze (Komada et al 2008)

Figure 9 Elevated plus maze apparatus

Mice were placed at the center of an apparatus facing open-arm and allowed to move freely on elevated plus maze apparatus (50 x 50 x 50 cm.) for 10 minutes. All movement was recorded by the video tracking system and analyzed by the software. The entry into each arm and total time spent at each arm were recorded as parameters of anxiety-like behavior.

4) Forced swimming test (Can et al 2012)

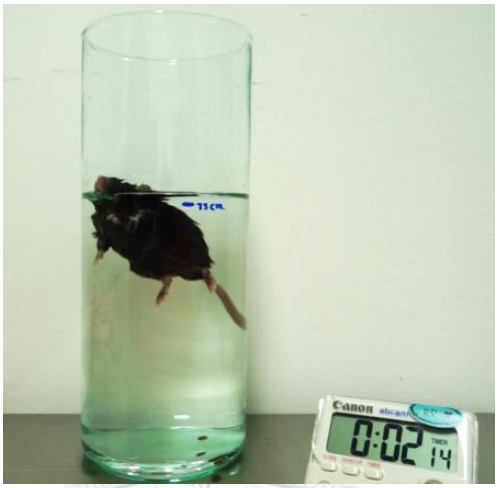


Figure 10 Forced swimming test apparatus

Mice were screened for depressive behavior by forced swimming test. The cylindrical tank (30 cm. height x 20 cm. diameter) was filled with room temperature water at 15 cm. height level. Mice were gently transferred into the tank and allowed to swim freely. The total time of the experiment was 6 minutes, which was separately analyzed as mobility and immobility time.

3.2.5 Nissl staining

Left hemisphere of brains from PND14, 6-week-old and 12-week-old mice was fixed in 4% paraformaldehyde until sectioning process. Brains were then transferred to 30% sucrose solution and immersed for 1-2 days. Brains were mounted onto the vibratome specimen disc with the frontal cortex facing the razor blade position. Frozen brains were sliced in coronal plane at -20 ºC by cryostat microtome at 10 µm thickness. Individual brain slices were placed on gelatin-coated glass slide. One brain section was picked from every 3 sections serially that covers DG, CA1 and CA3 area. Then, the slides were hydrated by immersing in 95% ethanol for 5 minutes, 70% ethanol for 5 minutes and running tap water for 1 minute. The slides were rinsed with distilled water for 30 seconds and immersed in 0.1% cresyl violet solution for 7 minutes. For differentiation, the slides were then immersed in 95% ethanol for 5

minutes. The slides were dehydrated by serially immersed in 95% ethanol for 1 minute, 100% ethanol for 1 minute, 100% ethanol for 5 minutes and xylene for 5 minutes. Purple-blue stained neurons were photographed using IX51® inverted microscope (Olympus America Inc., USA). All stained neurons in CA1 and CA3 were counted under 40X objective lens.

3.2.6 *In vitro* experimental design

Similar to behavioral studies, the pregnant mice were randomly divided into 2 groups which are control and MA-treated group. Mice in the control group received NSS 10 ml/kg by subcutaneous injection, while MA-treated mice received MA 5 mg/kg from GD8 to GD15. Pregnant mice were euthanized by CO_2 on GD16.5 to deliver embryos. Hippocampal tissues were dissected form the embryos to prepare primary cultures. The hippocampal neurons were incubated until DIV5, which was fixed and immunostained for neurite growth analysis. Immunofluorescent staining was also performed in DIV14 hippocampal neurons to evaluate synaptogenesis. Hippocampal neurons at DIV14 were harvested and used for western blot analysis. A schematic diagram of *in vitro* study is shown in Figure 11.

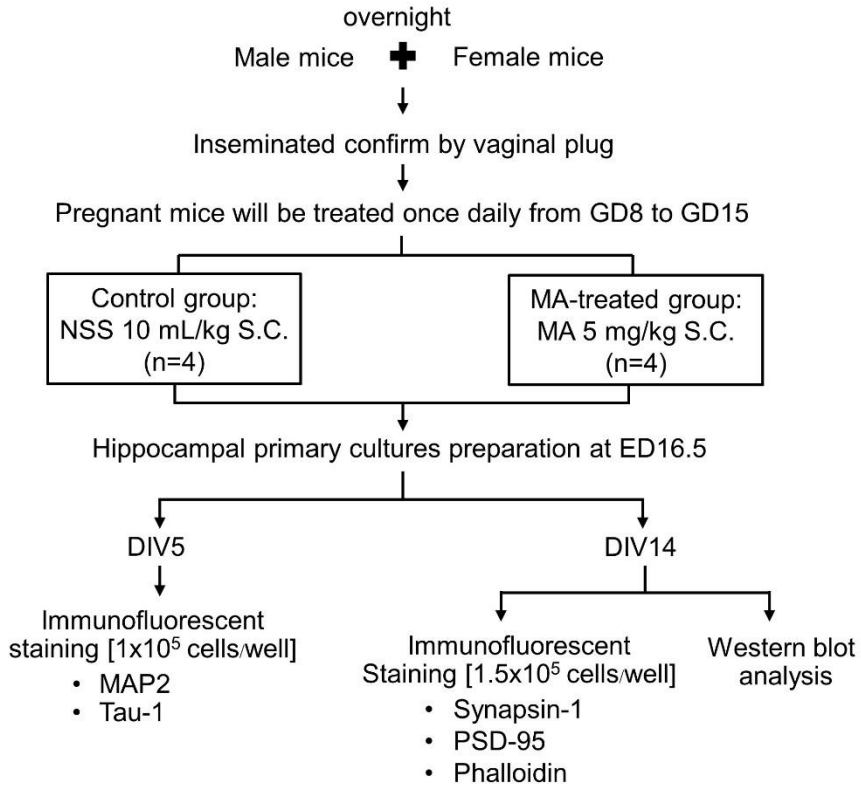


Figure 11 Schematic diagram of in vitro experiment protocol

3.2.7 Primary cell culture preparation (Pongrakhananon et al 2018)

Hippocampal primary neurons were obtained from hippocampal region of embryonic day 16.5 (ED16.5) brain. On GD16.5, pregnant mice were euthanized using CO_2 and confirmed death by decapitation. Abdominal wall was cleaned and cut open. Uterus was removed and placed in HCMF (HEPES buffer (100 mM), Ca^{2+}- and Mg^{2+}-free) buffer containing NaCl (1.37 M), KCl (54 mM), $Na_2HPO_4.7H_2O$ (34 mM), glucose (55.5 mM) and pyruvate (1:1000) in a petri dish on ice. Fetuses were decapitated. Then, skulls were disrupted, and brains were removed in a petri-dish containing HCMF buffer with pyruvate on ice. Brains were separated in sagittal plane, and hippocampi were dissected. The isolated hippocampi from 7 fetuses of the same litter were pooled and kept in cold HCMF buffer with pyruvate. The pooled hippocampi were then washed with HCMF buffer and digested by papain. Tissue pellets were incubated in DMEM supplemented with 5% horse serum, 5% fetal bovine serum, 1% penicillin/streptomycin, and deoxyribonuclease I for 1 minute and

washed again by DMEM. Tissue pellets were gently triturated through a pipette tip 6 times to dissociate a single cell. The cell suspension was centrifuged and resuspended twice in warm neurobasal medium supplemented with B-27, 0.25% glutamine and 1% penicillin/streptomycin. Cells were counted and plated on PDL-coated coverslip in 24-well plate at a density of densities of 1×10^5 and 1.5×10^5 cells/well to observe neuronal growth and synaptogenesis, respectively. For western blot analysis, cells were plated in 6-well plate. Primary hippocampal cultures were incubated at 37°C in a 5% humidified and 95% CO_2.

3.2.8 Western blot analysis

For *in vivo* study, tissues from each part of the brain were homogenized in RIPA lysis buffer with dounce homogenizer and remained on ice for 30 minutes. For *in vitro* study, primary hippocampal cultures at DIV14 were collected and lysed with lysis buffer on ice. Cell suspension was homogenized and left on ice for 30 minutes. Both cell lysate from tissue and cell culture were centrifuged at 16000g, 4°C for 20 minutes, and supernatant was collected. Protein concentration was determined by BCA protein Assay kit. Then, the supernatant was prepared for 40-80 µg protein and boiled in SDS-polyacrylamide gel electrophoresis (PAGE) sample loading buffer for 5 minutes. The sample preparations were electrophoresed by 10% SDS-PAGE at 80 V and electrotransferred onto a nitrocellulose filter membrane. The nonspecific proteins were blocked by incubating in 5% non-fat dried milk or 5% bovine serum albumin (BSA) in tris-buffered saline containing 0.05% tween-20 (TBST) for 1 hour at room temperature. Membranes were incubated at 4°C overnight with specific primary antibodies; anti-BDNF (1:200), anti-TrkB (1:400), anti-*p*-TrkB (1:1000), anti-CREB (1:1000), anti-*p*-CREB (Ser133) (1:1000), anti-synapsin-1 (1:8000), anti-PSD-95 (1:2000) and anti-GAPDH (1:1000). Membranes were washed with 0.075% TBST for 5 minutes, 3 times, and incubated with horseradish peroxidase–conjugated secondary immunoglobulin G (IgG) for 2 hours. After washing, membranes were visualized using electrochemiluminescence (ECL) detection reagent and captured using gel documentation system (Imagequant LAS 4000, GE Healthcare Life Sciences, UK). Image J software was used to analyze the density of the western blot bands.

3.2.9 Immunohistochemistry

To illustrate neuronal morphology, anti-MAP2 and anti-tau antibodies were used to stain dendrites and axons, respectively. On DIV5, hippocampal primary cultures on PDL-coated coverslips were fixed with warm 4% PFA in phosphate-buffered saline (PBS), and then incubated in 0.1% triton X-100. Fixed cells were blocked by 3% BSA before incubating with specific primary antibodies; anti-MAP2 (1:500) and anti-tau (1:2000), for 1 hour. After washing by PBS, secondary antibodies (Alexa Fluoro® 488 and Alexa Fluoro® 568) were added and incubated for 1 hour in dark. Hippocampal neurons were washed again with PBS mixing with DAPI for nuclei staining. Finally, coverslips were mounted on the glass slides and left at room temperature until dry. Synaptogenesis was evaluated from hippocampal primary cultures at DIV14 with the same preparation using 2% PFA, anti-synapsin I (1:2000) and anti-PSD95 (1:500) as primary antibodies for visualization of pre- and postsynaptic terminal, respectively.

Images of at least 50 neurons from each hippocampal culture were randomly captured and processed using a confocal fluorescent microscope (Leica TCS SP8 STED, Leica, Germany) with 40X and 100X oil immersion objective lens. Image J software was used to measure the thickness and length of an axon. The number of dendrites was measured as well. DIV14 neurons were randomly captured at least 15 fields from each hippocampal culture to quantify the number of presynaptic sites per 100 μm of axon length and also compute a size of overlap area between pre- and postsynapse.

3.3 Statistical analysis

A difference between groups were statistically analyzed using Independent t-test. All results were expressed as the mean ± S.E.M. The mean differences were considered to be statistical significance when P-value was less than 0.05.

CHAPTER IV

RESULTS

4.1 Effects of prenatal MA exposure on physical characteristics and neurobehaviors

4.1.1 Maternal body weight

The body weight of pregnant mice was observed during MA treatment period from GD8 to GD15. The average weight of the control and MA-exposed mice at GD8 was 25.26 ± 0.82 and 24.45 ± 0.88 g, respectively. The body weight of pregnant mice was gradually increased in both groups as shown in Figure 12. On the last day of treatment, GD15, an average weight of the control and MA-exposed mice was 35.08 ± 1.07 and 33.17 ± 1.11 g, respectively. The percentage of weight gain from GD8 to GD15 was calculated, which are 39.33 ± 3.27 and 35.89 ± 2.02 percent in the control and MA group, respectively. There was no significant difference of weight gain between the control and MA-exposed mice (Fig.13; $t(18) = 0.894$, $p = 0.3831$). This result indicated that MA treatment has no effect on maternal weight gain during pregnancy.

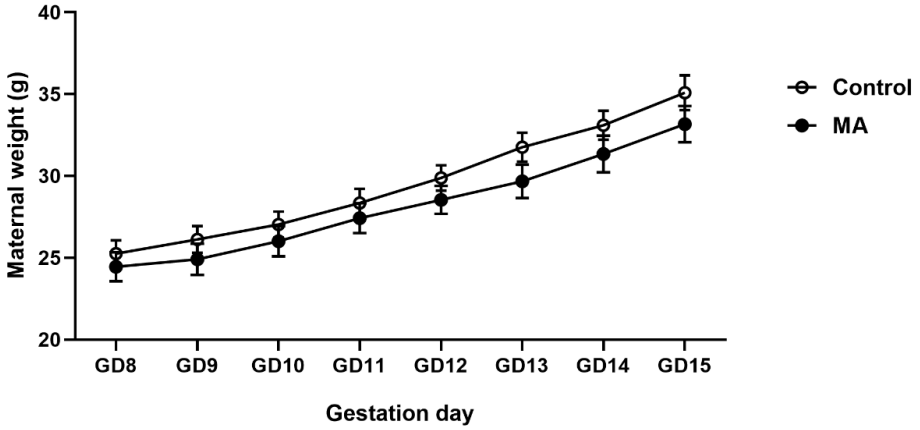


Figure 12 Body weight of pregnant mice during treatment period.
The data was expressed as the mean ± S.E.M. (n = 10/group).

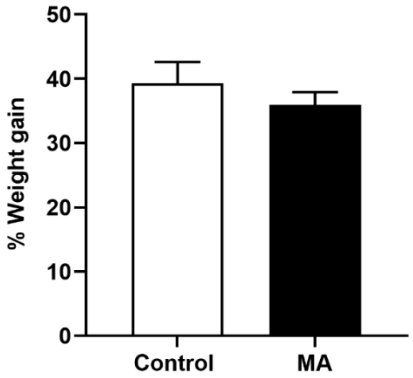


Figure 13 The percentage of maternal weight gain from GD8 to GD15.
The data was expressed as the mean ± S.E.M. (n = 10/group).

4.1.2 Body weight and morphology of the offspring

An average body weight of pups in all ages was described in table 1 as the mean ± S.E.M. The data in table 1 also showed a development of pups that was assessed by many morphological parameters. Crown-rump length was measured to represent body size, while head size was examined by Frontal-Occipital Length and Interparietal bone width, according to (Kawakami & Yamamura 2008) (Fig. 14). Exposure to methamphetamine in utero had an effect on the diameter of embryo head. At embryonic day 16.5, the diameter between occipital and frontal region of mice in MA-exposed group decreased significantly compared with that of the control mice ($t(61) = 5.323$, $p < 0.001$). The decreased frontal-occipital length was also observed in 12-week-old female mice exposed to MA in utero. There was no significant difference between 2 groups in weight and physical change of PND14, 6 weeks and 12 weeks old mice. Therefore, prenatal MA treatment revealed negative effect on physical development in embryonic age, but the effect was then presented only in adult female mice.

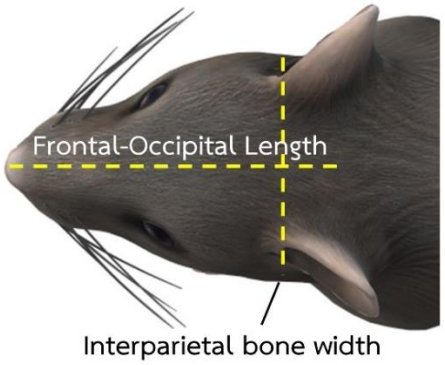


Figure 14 A measurement method of frontal-occipital length and interparietal bone width at mice head

Table 2 Body weight and morphological parameters of the offspring.

Mouse embryo, Day 16.5			
	Control	**MA-exposed**	***p*-value**
Crown-Rump length (mm)	15.71 ± 0.26, n=28	15.17 ± 0.21, n=35	0.1081
Frontal-Occ. Length (mm)	7.52 ± 0.11, n=28	***6.82 ± 0.08, n=35	<0.0001
Postnatal day 14			
	Control	**MA-exposed**	***p*-value**
weight (g)	6.83 ± 0.19, n=6	6.62 ± 0.49, n=6	0.7032
Crown-Rump length (mm)	47.5 ± 0.87, n=4	47.17 ± 1.4, n=6	0.8595
Frontal-Occ. Length (mm)	19.75 ± 0.29, n=5	19.36 ± 0.29, n=4	0.3710
Interparietal bone width (mm)	10.47 ± 0.51, n=5	10.97 ± 0.47, n=4	0.5063
week6			
Female	**Control**	**MA-exposed**	***p*-value**
weight (g)	15.91 ± 0.21, n=6	16.62 ± 0.27, n=6	0.0656
Crown-Rump length (mm)	68.83 ± 0.79, n=6	67.92 ± 0.84, n=6	0.4459
Frontal-Occ. Length (mm)	24.68 ± 0.26, n=4	25.2 ± 0.53, n=3	0.3745
Interparietal bone width (mm)	11.21 ± 0.36, n=6	11.48 ± 0.18, n=6	0.5126
Male	**Control**	**MA-exposed**	***p*-value**
weight (g)	18.51 ± 1.35, n=6	20.42 ± 0.75, n=6	0.2453
Crown-Rump length (mm)	72.92 ± 0.93, n=6	73 ± 0.58, n=6	0.9410
Frontal-Occ. Length (mm)	24.88 ± 0.59, n=4	25.02 ± 0.52, n=4	0.8568
Interparietal bone width (mm)	12.00 ± 0.18, n=6	11.65 ± 0.28, n=6	0.3076

week12			
Female	**Control**	**MA-exposed**	***p*-value**
weight (g)	22.11 ± 0.41, n=5	22.22 ± 1.14, n=5	0.9261
Crown-Rump length (mm)	74.04 ± 0.66, n=5	75.3 ± 1.68, n=5	0.5057
Frontal-Occ. Length (mm)	26.5 ± 0.23, n=5	*25.82 ± 0.16, n=5	0.0433
Interparietal bone width (mm)	12.18 ± 0.98, n=5	11.56 ± 0.16, n=5	0.5482
Male	**Control**	**MA-exposed**	***p*-value**
weight (g)	26.27 ± 0.6, n=7	27.4 ± 0.90, n=6	0.3190
Crown-Rump length (mm)	75.74 ± 0.65, n=7	76.33 ± 1.02, n=6	0.6247
Frontal-Occ. Length (mm)	26.64 ± 0.17, n=7	27.01 ± 0.29, n=6	0.2875
Interparietal bone width (mm)	11.64 ± 0.26, n=7	11.37 ± 0.24, n=6	0.4584

4.1.3 Locomotor activity

4.1.3.1 Adolescent mice

The distance traveled was measured over 5 minutes in the open-field test. The mean distances of adolescent female mice in the control and MA group were 2557 ± 227.7 and 2520 ± 66.01 cm, respectively. In adolescent male mice, the mean distances in the control and MA group were 2357 ± 204.7 and 2226 ± 161.5 cm, respectively. The results showed no significant difference between the control and treatment groups in both female and male mice (Fig. 15A; $t(10) = 0.1562$, $p = 0.8790$ and Fig. 15B; $t(10) = 0.5005$, $p = 0.6275$, respectively).

The locomotion time was also measured over 5 minutes in open-field test. The mean locomotion time of adolescent female mice in the control and MA group was 242.2 ± 6.096 and 251.5 ± 1.708 s, respectively. Male mice had locomotion time 244.7 ± 4.46 s in the control group and 242 ± 3.64 s in MA group. There was no significant difference between 2 groups in this locomotion time spent in adolescent mice (Fig. 15A; female; $t(10) = 1.474$, $p = 0.1712$ and Fig. 15B; male; $t(10) = 0.4630$, $p = 0.6533$).

The total distance traveled and locomotor time were also analyzed using two-way ANOVA with treatment and sex as main factors. There was no significant main effect of treatment ($F(1,20) = 0.2261$, $p = 0.6396$) and sex ($F(1,20) = 1.966$, $p =$

0.1762) on the distance traveled. Two-way ANOVA revealed no significant main effect of treatment ($F(1,20) = 0.6067$, $p = 0.4452$) and sex ($F(1,20) = 0.6689$, $p = 0.4231$) on locomotion time of adolescent mice as well.

4.1.3.2 Adult mice

At week 12, the average distances of female mice in the control and MA group were 2047 ± 421 and 1693 ± 127. 5 cm, respectively. Adult male mice exhibited the average distances as 1783 ± 157 and 1662 ± 161.7 cm for the control and MA group, respectively. Distance traveled of prenatal MA-exposed mice did not differ from the control mice in both female and male mice (Fig. 15C; $t(10) = 0.8043$, $p = 0.4400$ and Fig. 15D; $t(12) = 0.5291$, $p = 0.6064$, respectively).

The average time spent moving of female mice in the control and MA group was 230.8 ± 12.63 and 232.7 ± 13.64 s, respectively. Adult male mice spent time moving for 232.1 ± 5.02 and 236.0 ± 8.87 s in the control and MA group, respectively. Locomotion time of prenatal MA-exposed mice did not differ significantly from the control mice in both female and male mice (Fig. 15C; $t(10) = 0.09864$, $p = 0.9234$ and Fig. 15D; $t(12) = 0.4047$, $p = 0.6929$, respectively).

The total distance traveled and locomotor time were also analyzed using two-way ANOVA with treatment and sex as main factors. There was no significant main effect of treatment ($F(1,22) = 0.9972$, $p = 0.3289$) and sex ($F(1,22) = 0.3846$, $p = 0.5415$) on the distance traveled. Two-way ANOVA revealed no significant main effect of treatment ($F(1,22) = 0.08113$, $p = 0.7784$) and sex ($F(1,22) = 0.05326$, $p = 0.8196$) on locomotion time of adult mice as well.

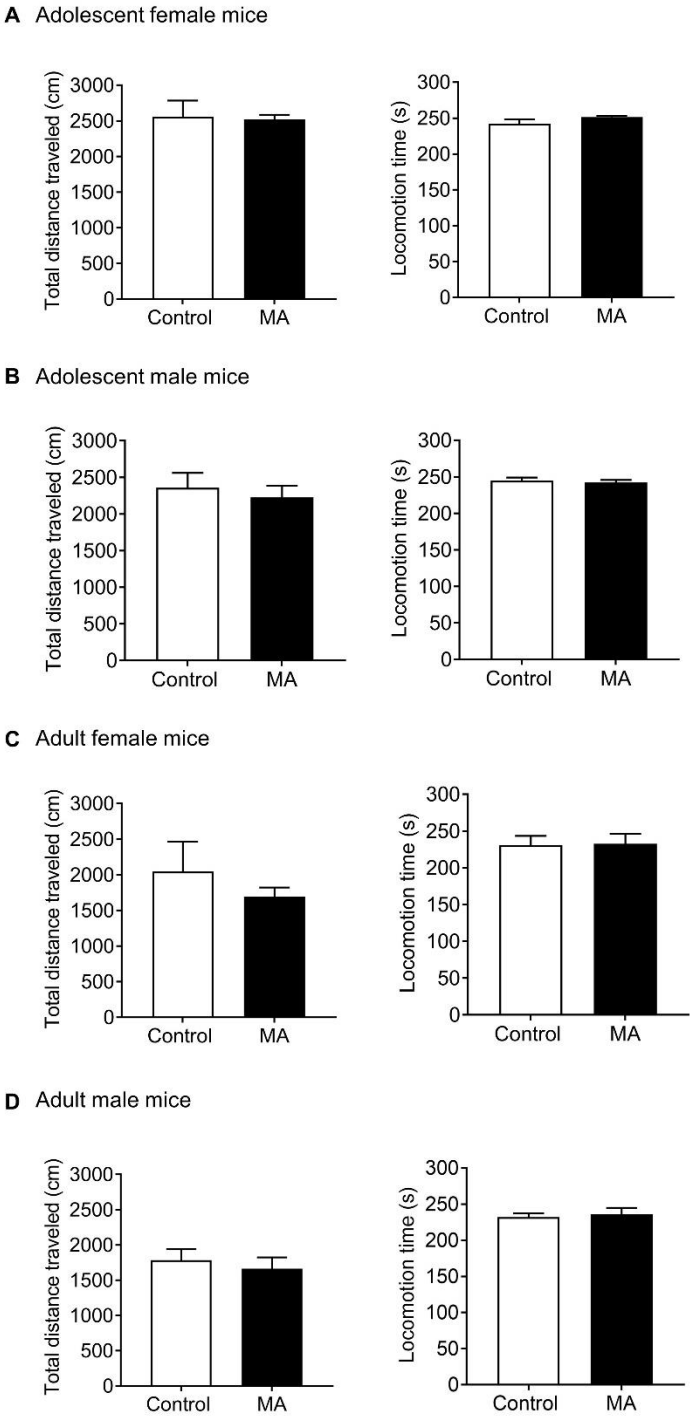


Figure 15 The total distances and locomotion time of open-field test

The total distances and locomotion time of adolescent female (A) and adolescent male mice (B), adult female (C) and adult male mice (D). All data are expressed as the mean ± S.E.M. (n = 6/group).

4.1.4 Recognition memory in the novel object recognition task

4.1.4.1 Adolescent mice

The discrimination index was used as a major parameter to evaluate working memory to recognize familiar and novel objects. Adolescent female mice revealed the discrimination index at 0.29 ± 0.06 in the control group and 0.18 ± 0.09 in MA group. Independent t-test showed no consequential effect of MA on memory in this group ($t(10) = 1.009$, $p = 0.3366$). The percent time spent exploring familiar or novel object was also measured to consider novel object recognition. As illustrated in Figure 16A, mice spent equal time exploring two objects in the training phase, while mice in the control group showed significantly greater preference on novel object (Paired $t(5) = 4.606$, $p = 0.0058$) in the test phase. Although MA mice failed to reach a significant difference between objects, a trend of novel object was displayed above 50% indicating novel object preference over familiar one. This result can assure that adolescent female mice can retain a recognition memory after an intertrial delay.

On the other hand, the discrimination index of adolescent male mice in the control and MA group was significantly different. The result showed that prenatal exposure of MA significantly reduced discrimination performance (Fig. 16B; $t(10) = 4.318$, $p = 0.0015$). This effect was also observed in global habituation when time spent exploring the familiar object was not different from that of the novel object in MA group as shown in Figure 16B. As a result, these findings indicated an effect of in utero MA exposure on recognition memory impairment was specific to adolescent male mice.

The discrimination index of adolescent mice from the test phase was analyzed by two-way ANOVA, which revealed the significant effect of treatment on the discrimination index ($F(1,20) = 12.96$, $p < 0.01$). There was no effect of sex and treatment x sex interaction on discrimination index in adolescent mice. These results suggested that MA-exposed adolescent mice showed recognition memory impairment without the inference from sex factor.

4.1.4.2 Adult mice

An effect of prenatal MA exposure on working memory of adult mice was in the same direction among two sexes. The discrimination index of 12-week-old female mice was 0.34 ± 0.06 and 0.12 ± 0.06 in the control and MA group, respectively. The unpaired t-test demonstrated a significant difference between the control and MA group ($t(10) = 2.46$, $p = 0.0337$). A distinction in the preference of two objects was observed merely in the control group at the test phase (Fig. 16C).

The discrimination index of adult male mice in the control and MA group was 0.36 ± 0.06 and 0.12 ± 0.043, respectively. The mean discrimination index of MA group was significantly lower than that of the control mice ($t(10) = 3.257$, $p = 0.0086$). Figure 16D illustrated a discrepancy in the preference of the control mice and also unexpecting MA group in the testing phase. (Control; Paired $t(5) = 5.76$, $p = 0.0022$ and MA; Paired $t(5) = 2.68$, $p = 0.0437$, novel vs familiar). Taken together, prenatal exposure of MA notably affected adverse memory alterations, especially working memory in adult age.

Two-way ANOVA was performed with treatment and sex as main factors on the discrimination index of adult mice. Two-way ANOVA revealed the significant effect of treatment on the discrimination index ($F(1,20) = 15.81$, $p < 0.001$). There was no effect of sex and treatment x sex interaction on discrimination index in adult mice. These results suggested recognition memory impairment in adult mice of MA group in female and male mice.

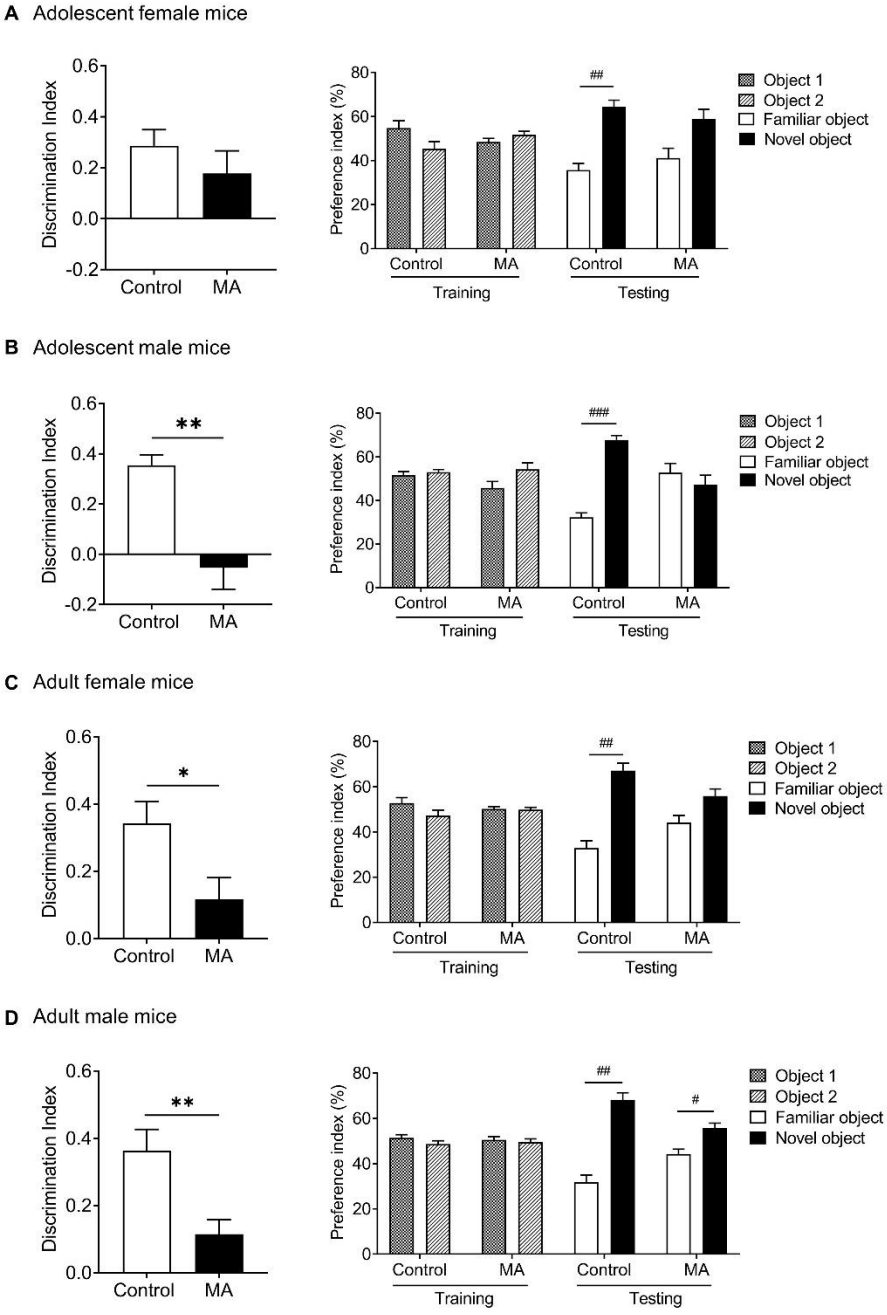


Figure 16 Effects of prenatal MA exposure on NOR parameters

The discrimination index and percentage of preference index on familiar and novel object in the training and testing phases of adolescent female (A) and adolescent male mice (B), adult female (C) and adult male mice (D) are calculated as a parameter of recognition memory. All data are expressed as the mean + S.E.M. (n = 6/group). *$p < 0.05$, **$p < 0.01$ control vs MA exposure, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ novel vs familiar

4.1.5 Spatial memory in the novel location test

4.1.5.1 Adolescent mice

Another memory function was investigated through location novelty, so the discrimination index was used to emerge a discrimination behavior between two places. In adolescent group, the discrimination index of female mice was 0.28 ± 0.07 in the control group versus 0.09 ± 0.04 in MA group. Location discrimination performance of MA-exposed mice was significantly lower than that of the control mice ($t(10) = 2.47$, $p = 0.0332$). The preference index of familiar and novel location was not different in the training phase. While the control mice had a distinct preference for both locations (Paired $t(5) = 4.09$, $p = 0.0095$), MA group could not remain a memory of familiar location in the testing phase (Fig. 17A).

On the contrary, male mice showed no significant effect of MA treatment on the discrimination index (control; 0.36 ± 0.06 versus MA; 0.21 ± 0.11) as illustrated in Figure 17B ($t(10) = 1.28$, $p = 0.2299$). The result showed that the control mice spent more time exploring object in the novel location than object in the familiar location (Paired $t(5) = 6.180$, $p = 0.0016$). Even though MA-exposed mice failed to reach a significant difference between locations during the testing phase, there was a trend with increases in preference of novelty over familiar location.

The discrimination index of adolescent mice from the NLT model was analyzed by two-way ANOVA, which revealed the significant effect of treatment on the discrimination index ($F(1,20) = 5.884$, $p < 0.05$. There was no effect of sex and treatment x sex interaction on discrimination index in adolescent. These results suggested spatial memory impairment in adolescent mice of MA group without the inference from sex factor.

4.1.5.2 Adult mice

Over time, a vulnerable effect on memory was noteworthy in adult mice. MA treatment during pregnancy significantly decreased the discrimination index of female mice at 12- week-old (control; 0.399 ± 0.07 versus MA; 0.01 ± 0.09; $t(9) = 3.543$, $p = 0.0063$). Percent of time spent exploring each location in both phases also confirmed

the effect of MA, which MA-exposed mice preferred the objects in the novel and familiar locations equally after inter-trial delay (Fig. 17C).

The outcome from adult male mice was consistent with those of adult female mice which MA-exposed mice had a significantly lower discrimination index ($t(10) = 3.79$, $p = 0.0035$) than the control mice (Fig. 17D). According to exploration time profile, MA-exposed mice had an equal preference on the objects in the novel and familiar location after the training phase.

Two-way ANOVA was also performed with treatment and sex as main factors on the discrimination index of adult mice. Two-way ANOVA revealed the significant effect of treatment on the discrimination index ($F(1,19) = 25.91$, $p < 0.001$). There was no effect of sex and treatment x sex interaction on discrimination index in adult mice. Taken together, the results from two-way ANOVA suggested that MA induced spatial memory impairment in both ages.

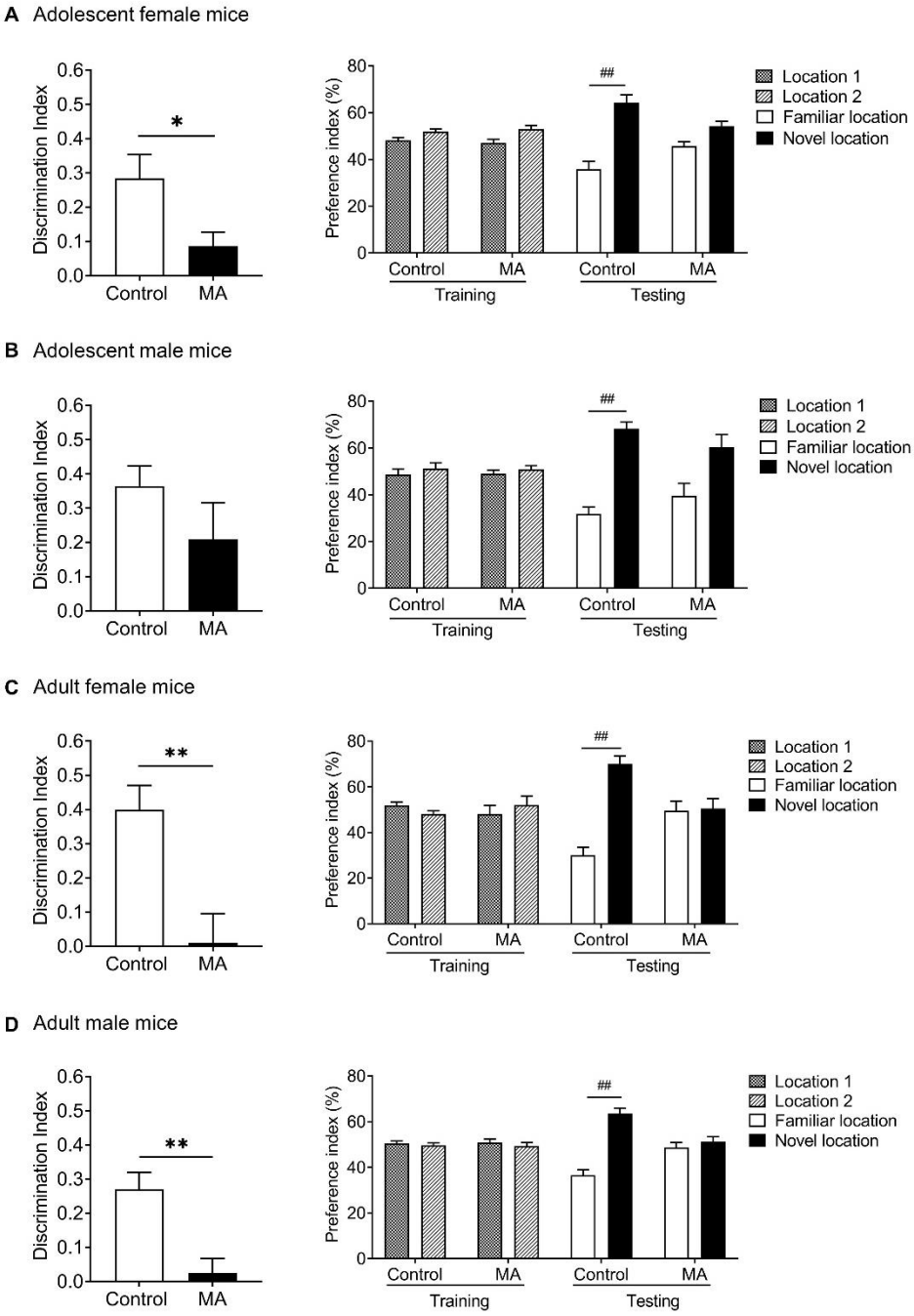


Figure 17 Effects of prenatal MA exposure on NLT parameters

The discrimination index and percent of preference index on familiar and novel location in the training and testing phases of adolescent female (A) and adolescent male mice (B), adult female (C) and adult male mice (D) are calculated as a parameter of spatial-related memory. All data are expressed as the mean ± S.E.M. (n = 6/group). *$p < 0.05$, **$p < 0.01$ control vs MA exposure, $^{\#\#}p < 0.01$ novel vs familiar

4.1.6 Anxiety-like behavior

To determine an anxiety status, total open arm entries and time spent in each arm were recorded in the elevated plus maze. The total entries into open arms of adolescent female and male mice were 19.00 ± 3.11 (control), 18.50 ± 4.26 (MA) and 10.50 ± 1.18 (control), 10.33 ± 2.67 (MA), respectively. There was no significant difference in the number of open arm entries (Fig. 18A; Female; $t(10) = 0.095$, $p = 0.9263$ and Fig. 18B; male; $t(10) = 0.05718$, $p = 0.9555$). The preference for being in each arm was exhibited in Figure 18A-B, which appeared in the same direction between both control and MA group.

At week 12, the average number of total entries into open arms for adult female and male mice was 14.33 ± 1.80 (control), 11.17 ± 1.97 (MA) and 12.33 ± 1.69 (control), 12.50 ± 1.688 (MA), respectively. There was no significant difference in the number of open arm entries (Fig. 18C; Female; $t(10) = 1.18$, $p = 0.2633$ and Fig. 18D; male; $t(10) = 0.07$, $p = 0.9457$). Similar to adolescent mice, the preference for being in each arm was not different between treatment groups. These results indicated no anxiogenic effect of MA exposure during pregnancy in every age stage.

Effects of sex on time spent in open arm were analyzed by two-way ANOVA with treatment and sex as main factors. In adolescent mice, there was no significant main effect of sex ($F(1,20) = 3.230$, $p = 0.0874$) and treatment ($F(1,20) = 0.8995$, $p = 0.3542$) factor. Two-way ANOVA also revealed no significant main effect of sex ($F(1,20) = 0.1810$, $p = 0.6751$) and treatment ($F(1,20) = 0.4677$, $p = 0.5019$) factor in adult mice. Taken together, prenatal MA exposure did not induce anxiety in adolescent and adult mice with no sex difference.

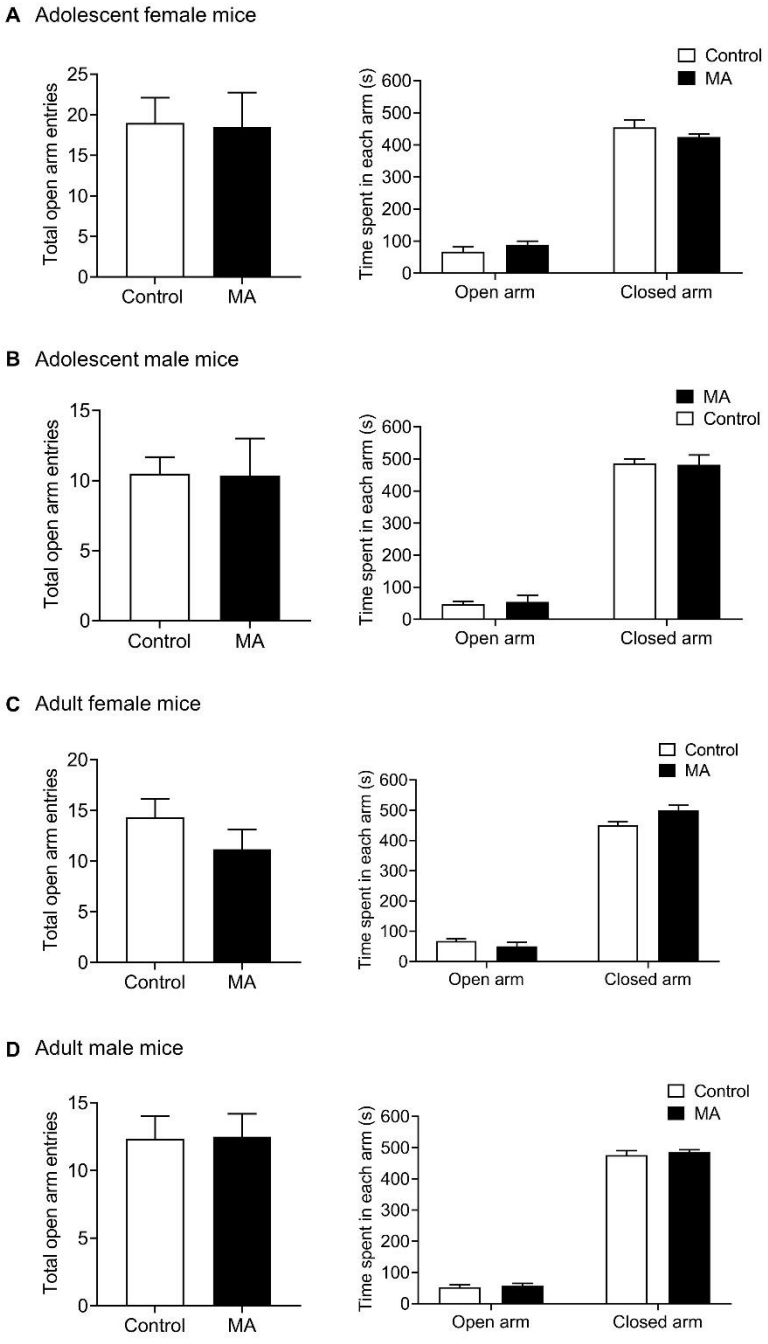


Figure 18 The total open arm entries and time spent in each arm of elevated plus maze

The total open arm entries and time spent in each arm in the elevated plus maze of adolescent female (A) and adolescent male mice (B), adult female (C) and adult male mice (D). All data are expressed as the mean ± S.E.M. (n = 6/group).

4.1.7 Depression-like behavior

Forced swimming test was used to assess depression-like behavior. At week 6, the average immobility time of female mice in the control and MA group were 269.7 ± 17.89 and 265.4 ± 15.13 s, respectively. Immobility time of male mice were 268.9 ± 12.28 s in the control group and 257.7 ± 16.8 s in MA-exposed group. Independent t-test presented no significant difference in the immobility time between groups in both female and male (Fig. 19A; $t(9) = 0.19$, $p = 0.855$ and Fig. 19B; $t(10) = 0.54$, $p = 0.6019$, respectively).

At week 12, the mean immobility time of female mice in the control and MA group were 239.3 ± 28.56 and 276.3 ± 10.39 s, respectively. Male mice displayed the immobility time as 242.4 ± 15.07 s in the control group and 261.0 ± 9.48 s in MA-exposed group. There was no significant difference observed between groups in both sexes (Female; $t(10) = 1.22$, $p = 0.2509$ and male; $t(12) = 0.96$, $p = 0.3548$). Therefore, prenatal MA exposure did not alter the levels of depressive behavior in both sexes at later age (Fig. 19C-D).

For assessment of sex effect, two-way ANOVA with treatment and sex as main factor was performed on immobility time. There was no significant main effect of sex ($F(1,19) = 0.07456$, $p = 0.7878$) and treatment ($F(1,19) = 0.2509$, $p = 0.6222$) on immobility time of adolescent mice. Two-way ANOVA of adult mice also revealed no significant main effect of sex ($F(1,22) = 0.1212$, $p = 0.7311$) and treatment ($F(1,22) = 2.530$, $p = 0.1260$) on immobility time. Therefore, MA showed no long-term effect on depressive level of adolescent and adult mice without sex differences.

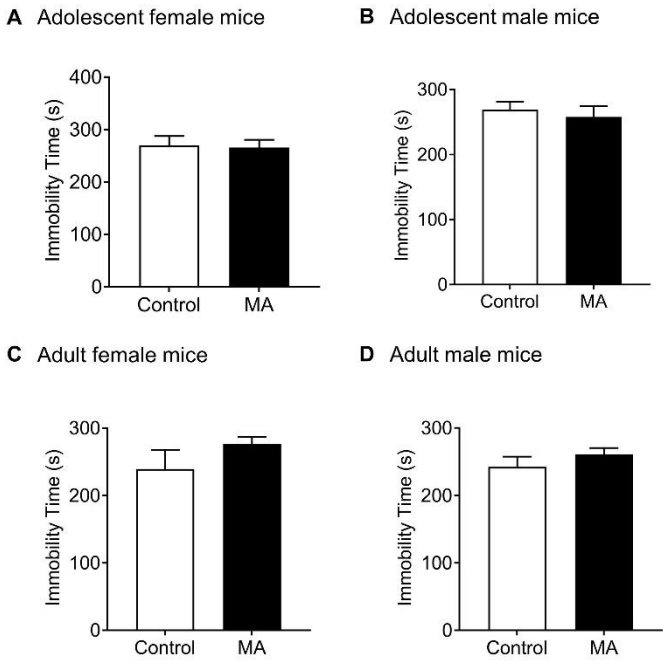


Figure 19 Immobility time of forced swimming test

Immobility time of adolescent female (A) and adolescent male mice (B), adult female (C) and adult male mice (D). All data are expressed as the mean ± S.E.M. (n = 6/group).

4.2 Effects of prenatal MA exposure on neuronal structure

4.2.1 Neuronal morphology of the primary hippocampal neurons

The isolated hippocampal neurons from prenatal MA-exposed embryos showed the detrimental outcomes in both dendrites and axons. The secondary dendrites were identified as branches that emerged from primary dendrites (Fig. 20A). The secondary dendrites were quantified in order to investigate an effect on dendrite outgrowth. The average number of secondary dendrites of hippocampal neurons in the control and MA groups was 2.78 ± 0.14 and 2.34 ± 0.16, respectively. The number of secondary dendrites was significantly diminished in MA group compared to the control cells (Fig. 20B; $t(429) = 2.034$, $p = 0.0426$)

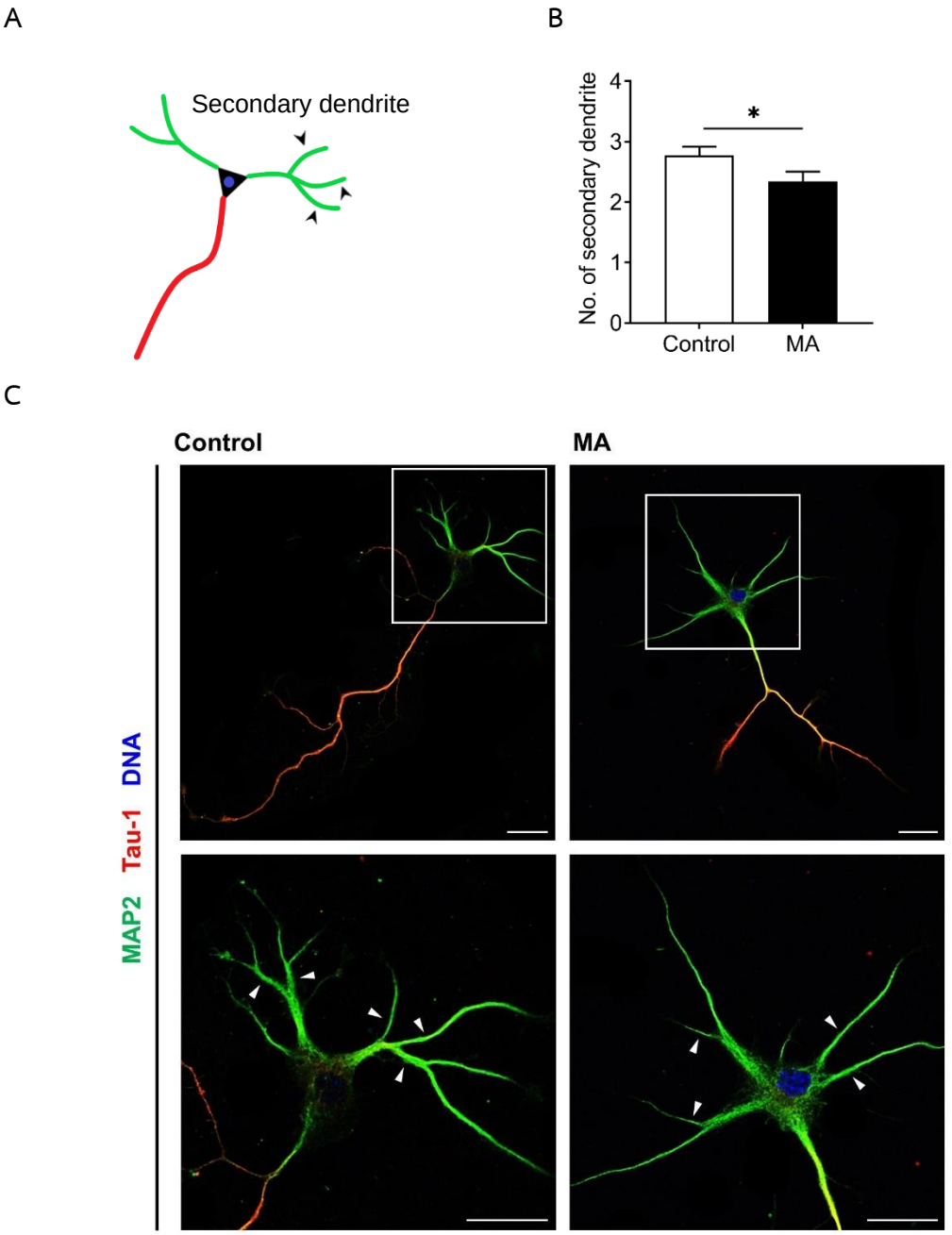


Figure 20 Effects of MA on dendrite outgrowth of the primary hippocampal neurons (A) Schematic drawing illustrates the color staining of dendrite marker (MAP2; green), axon marker (Tau-1; red) and DNA (blue) (B) The number of secondary dendrites (white arrow) was measured and plotted as mean ± S.E.M. *$p < 0.05$ vs control group. (n (control, MA) = 236, 195). (C) Primary hippocampus neurons at DIV5 were immunostained with antibody to dendrite, axon and nucleus. Box areas are enlarged at the bottom panel. Scale bar = 25 μm

To examine whether axons were also altered by MA exposure, the structural features were assessed. Axon length of the control and MA group were 183.3 ± 3.93 and 143.6 ± 4.45 μm, respectively, as shown in Figure 20B. Axonal diameter was measured at the widest area of that axon, which was 2.27 ± 0.05 and 1.52 ± 0.04 μm for the control and MA group, respectively (Fig. 21C). Both length and diameter of axons were significantly reduced after prenatal MA exposure ($t(441) = 6.7$, $p < 0.001$, $t(441) = 11.76$, $p < 0.001$, respectively). Taken together, these results indicated that the neurite growth of hippocampal neurons was influenced by MA exposure during embryonic development.

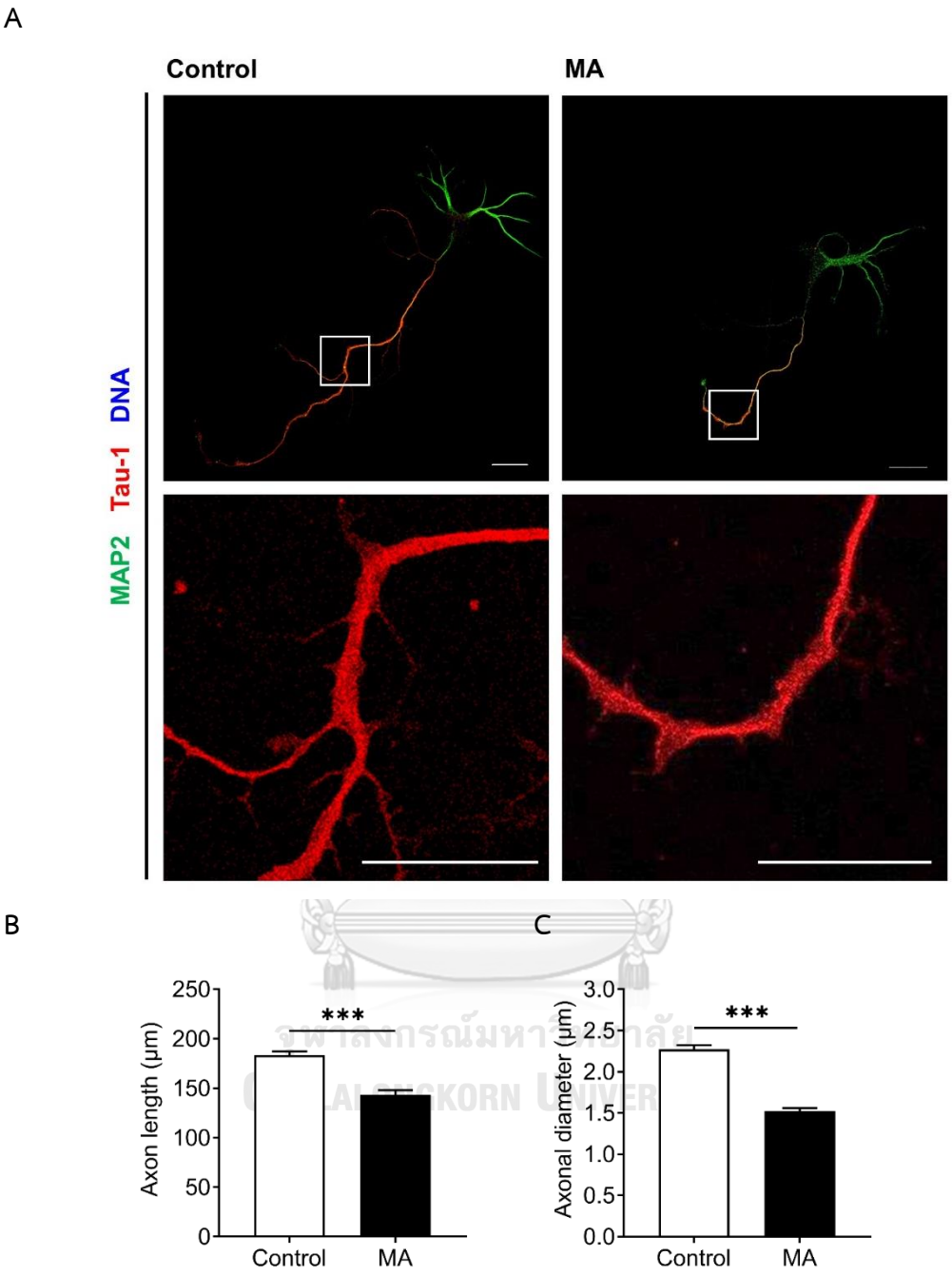


Figure 21 Effects of MA on axon length and diameter of the primary hippocampal neurons

(A) Primary hippocampus neurons at DIV5 were immunostained with antibody to dendrite marker (MAP2; green), axon marker (Tau-1; red) and DNA (blue). Box areas are enlarged at the bottom panel. (Scale bar = 25 μm) Axon length (B) and diameter (C) were measured and plotted as mean ± S.E.M. ***$p < 0.001$ vs control group (n (control, MA) = 244, 199)

4.2.2 The quantification of presynaptic protein cluster in the primary hippocampal neurons

Synaptic terminal is essentially responsible for the functions of synaptic transmission. Presynaptic terminal, which dominantly locates on axons, was assessed since axon morphology changed after prenatal MA exposure. The immunofluorescent imaging of synapsin-1 as the presynaptic marker was shown by green dot-like boutons (Fig. 22A-B). The average number of presynaptic sites per 100 μm-axon length in the control and MA group was 33.17 ± 1.43 and 17.85 ± 0.83, respectively. MA administration significantly decreased the extent of presynaptic terminals in cultured hippocampal neurons (Fig. 22C; $t(128) = 8.805$, $p < 0.001$).

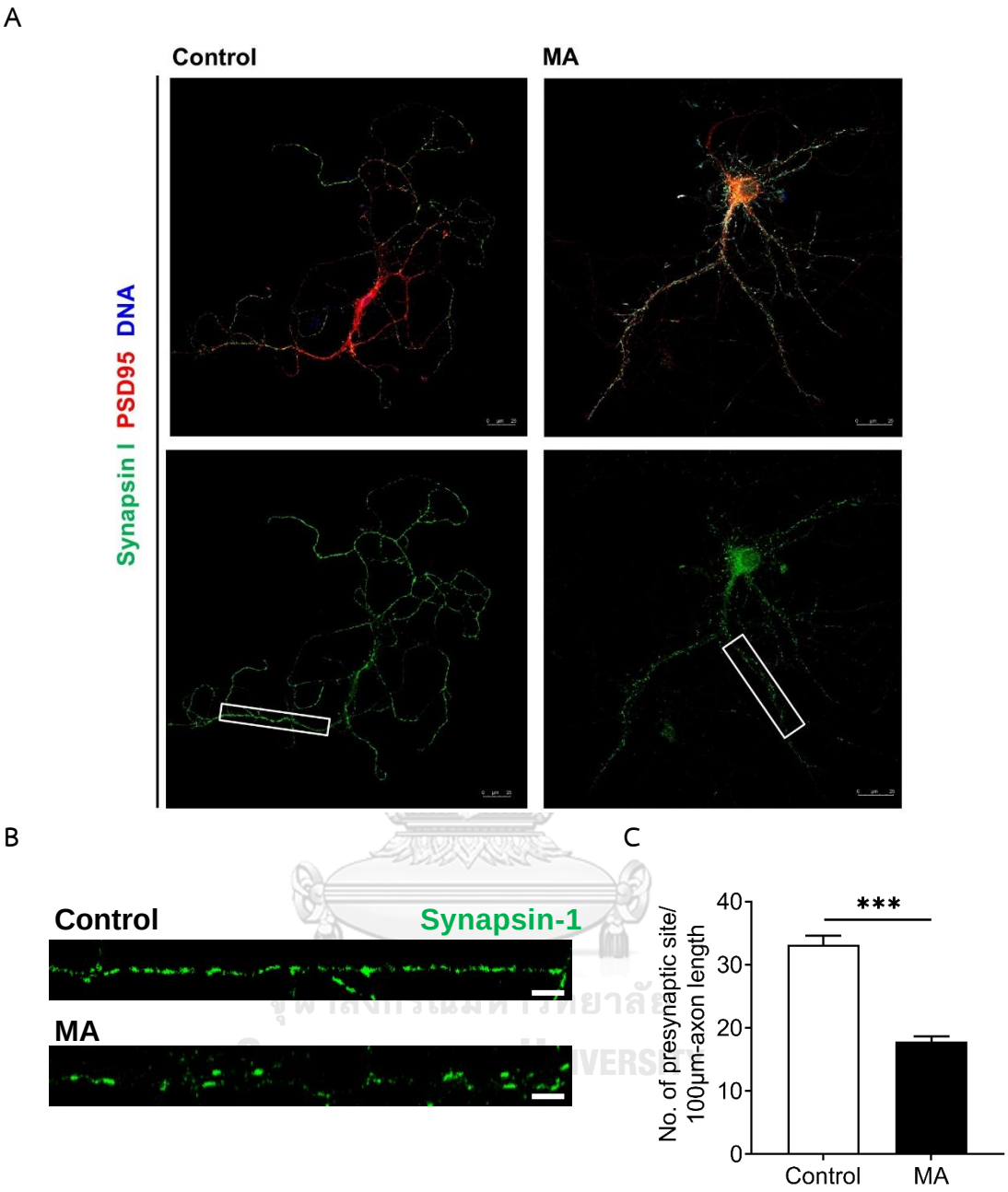


Figure 22 Effects of MA on the number of presynaptic terminals in the primary hippocampal neurons

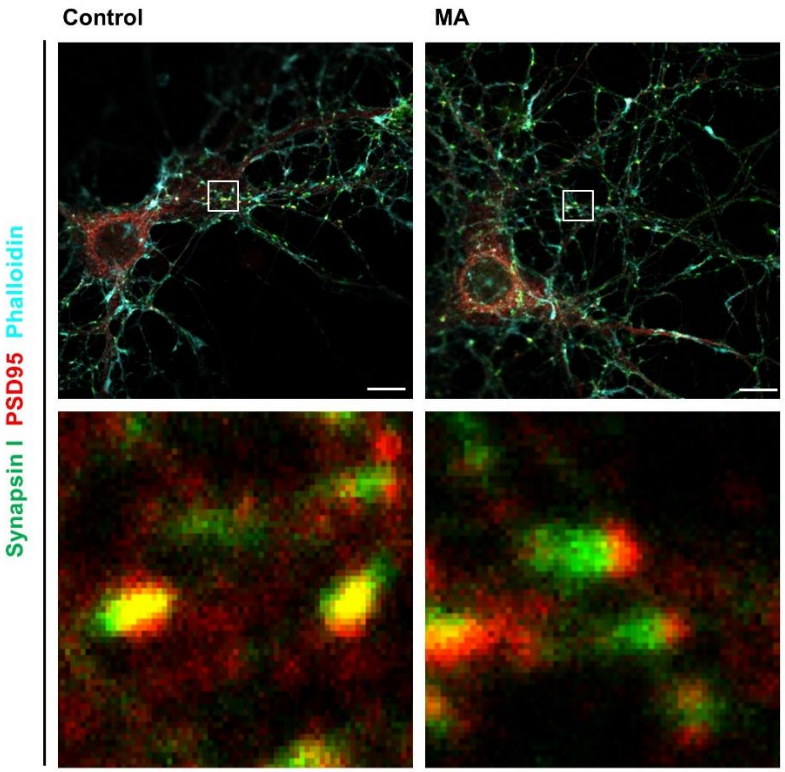
(A) Primary hippocampus neurons at DIV14 were immunostained with antibody to presynaptic marker (synapsin-1; green), postsynaptic marker (PSD95; red) and phalloidin (blue). (scale bar = 25 µm). (B) Box areas are enlarged to show high magnificant image of primary hippocampus culture at DIV14 labelled with antibody to synapsin-1 (green). (Scale bar = 5 µm) (C) The number of presynaptic site was

counted and plotted. The data are presented as the mean ± SEM. ***$p < 0.001$ vs control group (n (control, MA) = 71, 59)

4.2.3 Colocalization-based synapse detection in the primary hippocampal neurons

After axon growing and synaptic protein cluster expression, synapse formation processes to accomplish neuron communication. Therefore, the contact area between pre- and postsynaptic puncta was measured to investigate the completion of synapse formation. The area of colocalized synaptic markers in the control and MA group were 0.10 ± 0.0036 and 0.04 ± 0.0016 μm^2, respectively. Colocalization of synaptic protein markers was significantly smaller in MA-exposed group as compared to the control (Fig. 23B; $t(338) = 14.86$, $p < 0.001$). These results suggested that MA induced a loss of presynapse and impaired synaptogenesis which might lead to uncompleted synaptic function.

A



B

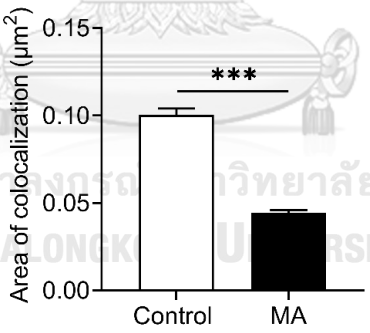


Figure 23 The analysis of co-localization between pre- and postsynaptic in the primary hippocampal neurons

(A) Primary hippocampus neurons at DIV5 were immunostained with antibody to presynaptic marker (synapsin-1, green), postsynaptic marker (PSD95, red) and actin (phalloidin, blue). (Scale bar = 8 μm) (B) Box areas are enlarged presenting synaptic boutons. (C) Co-localization area between synapsin-1 and PSD95 (yellow area) was quantified. The data are the mean ± SEM. ***$p < 0.001$ vs control group (n (control, MA) = 161, 179)

4.2.4 Synaptic protein expression in the hippocampal tissue

To study synaptic development, western blot technique was also used to analyze the expression of synaptic proteins; PSD-95 and synapsin-1 as the parameters of pre- and postsynapse, respectively. Only hippocampal tissue was considered here as a substantial area of connections between synapses and memory. In PND14 mice, the expression levels of synapsin-1 decreased significantly in MA-exposed mice ($t(7) = 2.660$, $p = 0.0325$), while PSD-95 levels in MA-exposed mice were not different from that of the control mice (Fig. 24A; $t(8) = 0.2123$, $p = 0.8372$). This outcome indicated that MA exposure in embryo could have repercussions for presynaptic development at an early age.

In order to notice the negative effects in later age, the hippocampus from the adolescent mice was investigated and presented in Figure 24B for females and males. It was shown that the expression levels of PSD-95 in MA-exposed were higher than those of the control mice in female ($t(9) = 2.479$, $p = 0.0350$) and male mice ($t(10) = 2.846$, $p = 0.0174$). However, synapsin-1 expression levels did not differ from one another. Therefore, a consequence of MA exposure mainly attracted to postsynapse over presynapse in adolescence, which contrasted with those in early age.

Subsequently, an influence of MA for adult female mice was prevalent. MA-exposed adult female mice had significantly lower expression levels of synapsin-1 ($t(9) = 3.301$, $p = 0.0092$), but higher PSD-95 expression levels than the control mice (Fig. 24C; $t(10) = 3.421$, $p = 0.0065$). However, increases in PSD-95 protein expression were observed in MA-exposed adult male mice ($t(9) = 2.370$, $p = 0.0419$), but the expression levels of synapsin-1 were not different (Fig. 24C). These results indicated that MA was likely to eradicate an existence of presynapse, whereas postsynaptic protein collectively had an opposite tendency.

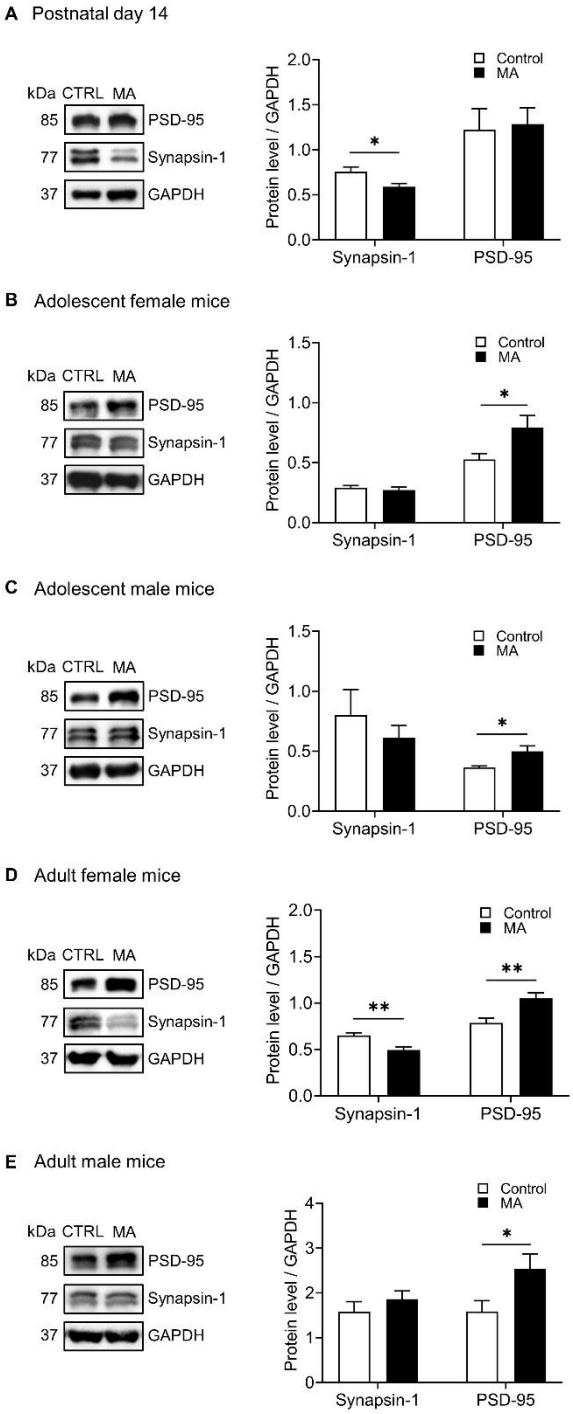


Figure 24 The expression levels of synaptic proteins in the hippocampus Western blot analysis of synapsin-1 and PSD-95 expression in the hippocampus of postnatal day 14 mice (A), adolescent female (B) and adolescent male mice (C), adult female (D) and adult male mice (E). The band intensity was quantified and normalized to GAPDH. The data are illustrated as the mean ± S.E.M. *$p < 0.05$, **$p < 0.01$ vs control group. (n = 6/group)

4.2.5 The number of neurons in the hippocampus

Neurons with nissl staining were counted in 2 areas of the hippocampus. In CA1 area of PND14 mice, the number of neurons was 79.20 ± 2.284 and 95.20 ± 9.467 for the control and MA group, respectively. Hippocampus's CA3 area of PND14 mice had 129.4 ± 8.224 neurons in the control mice and 130.3 ± 7.123 neurons in MA-exposed mice. Independent t-test showed no significant difference of the number of neurons between 2 groups in CA1 ($t(8) = 1.643$, $p = 0.1390$) and CA3 ($t(8) = 0.08272$, $p = 0.9361$) areas (Fig. 25B).

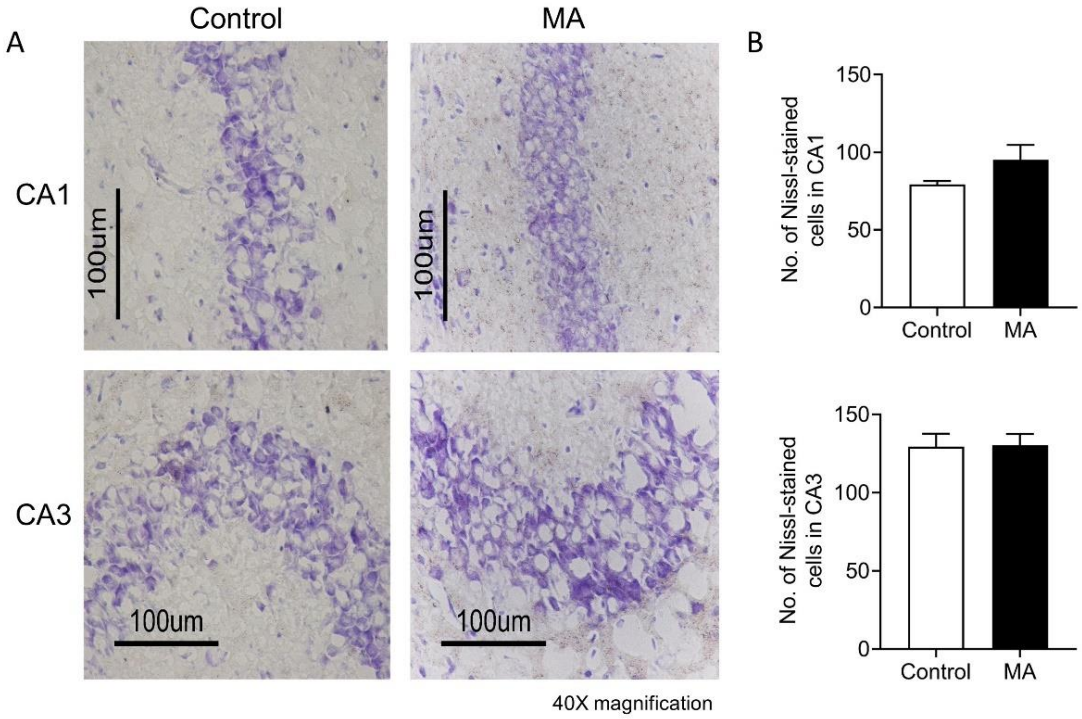


Figure 25 The number of neurons in the hippocampal CA1 and CA3 of PND14 mice (A) Nissl-stained neuron. The microscopic image was displayed in 40X magnification. (B) The number of nissl-stained cells was presented as the mean ± S.E.M. (n = 6/group).

In adolescent female mice, the number of neurons in CA1 and CA3 areas was 65.90 ± 3.4; control vs 74.20 ± 1.34; MA and 130.5 ± 4.45; control vs 128.5 ± 3.73; MA, respectively (Fig. 26B). In adolescent male mice, the number of neurons in CA1 and CA3 areas was 104.4 ± 3.08; control vs 99.92 ± 5.18; MA and 153.6 ± 3.75; control vs 151.4 ± 7.22; MA, respectively (Fig. 27B). The number of neurons in MA-treated group was not significant different from the control in both areas and both sexes.

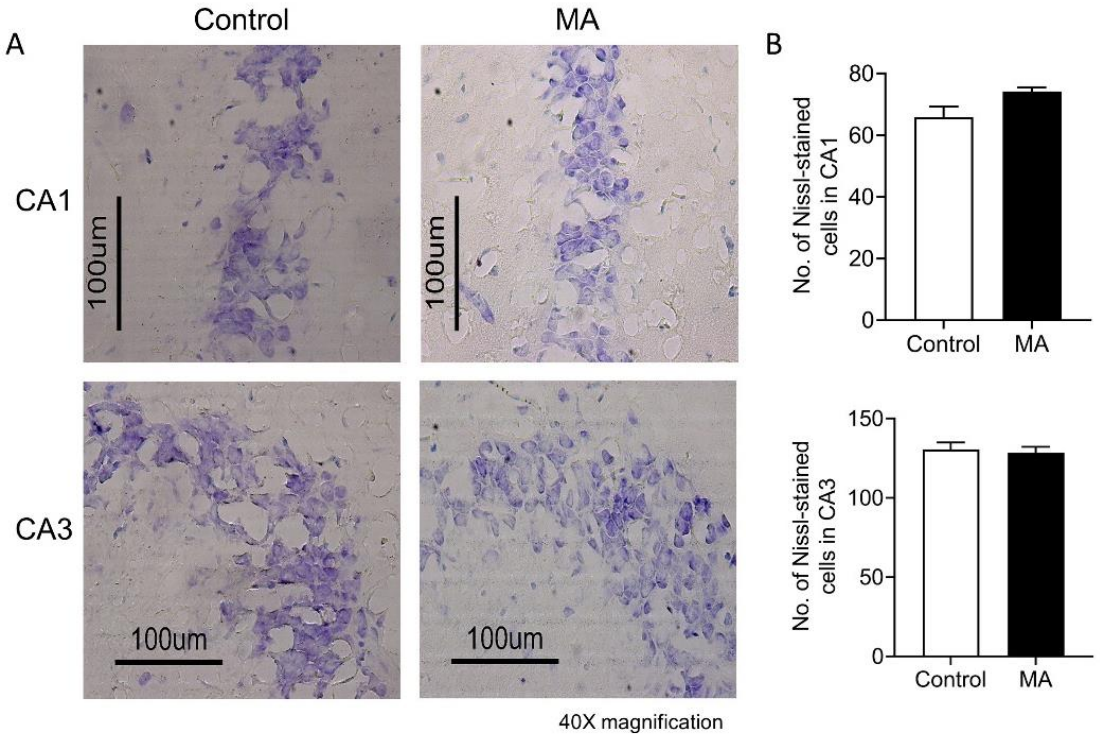


Figure 26 The number of neurons in the hippocampal CA1 and CA3 of adolescent female mice (6 weeks old)

(A) Nissl-stained neuron. The microscopic image was displayed in 40X magnification.

(B) The number of nissl-stained cells was presented as the mean ± S.E.M. (n = 6/group).

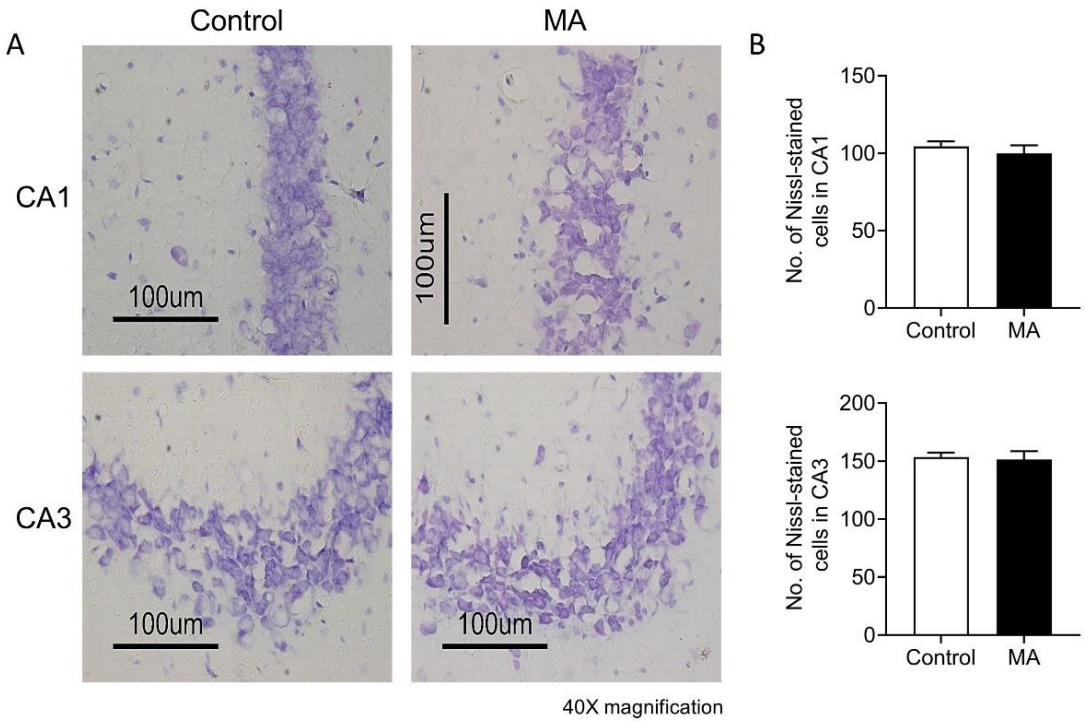


Figure 27 The number of neurons in the hippocampal CA1 and CA3 of adolescent male mice (6 weeks old)

(A) Nissl-stained neuron. The microscopic image was displayed in 40X magnification.

(B) The number of nissl-stained cells was presented as the mean ± S.E.M. (n = 6/group).

The number of neurons in 12-week-old mice was also assessed using nissl staining in tissue sections, which presented in Figure 28-29. Results from adult mice were in the same manner as infantile and adolescent mice. In adult female mice, the number of neurons in CA1 brain slice was 95.83 ± 3.12 and 103.0 ± 3.69 cells, while those in CA3 area was 136.2 ± 4.42 and 135.4 ± 4.45 cells in control and MA group, respectively (Fig. 28B). Adult male mice had 94.83 ± 5.99 and 84.75 ± 3.55 cells in CA1, and 141.3 ± 7.74 and 130.8 ± 4.36 cells in CA3, in the control and MA group, respectively (Fig. 29B).

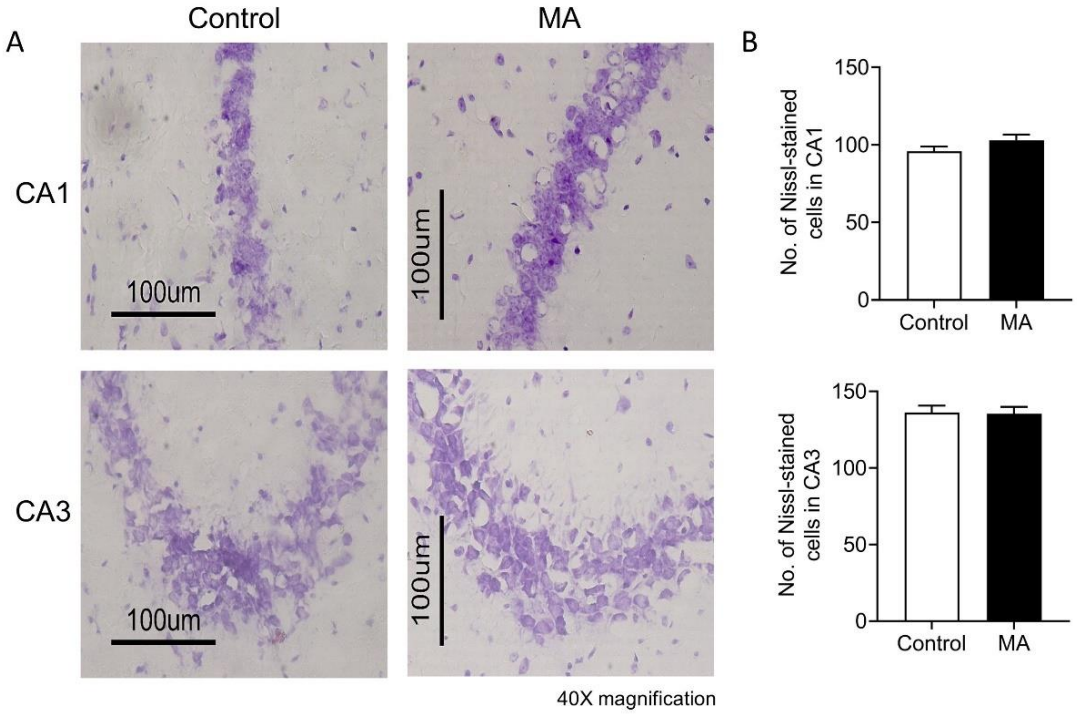


Figure 28 The number of neurons in the hippocampal CA1 and CA3 of adult female mice (12 weeks old)

(A) Nissl-stained neuron. The microscopic image was displayed in 40X magnification.

(B) The number of nissl-stained cells was presented as the mean ± S.E.M. (n = 6/group).

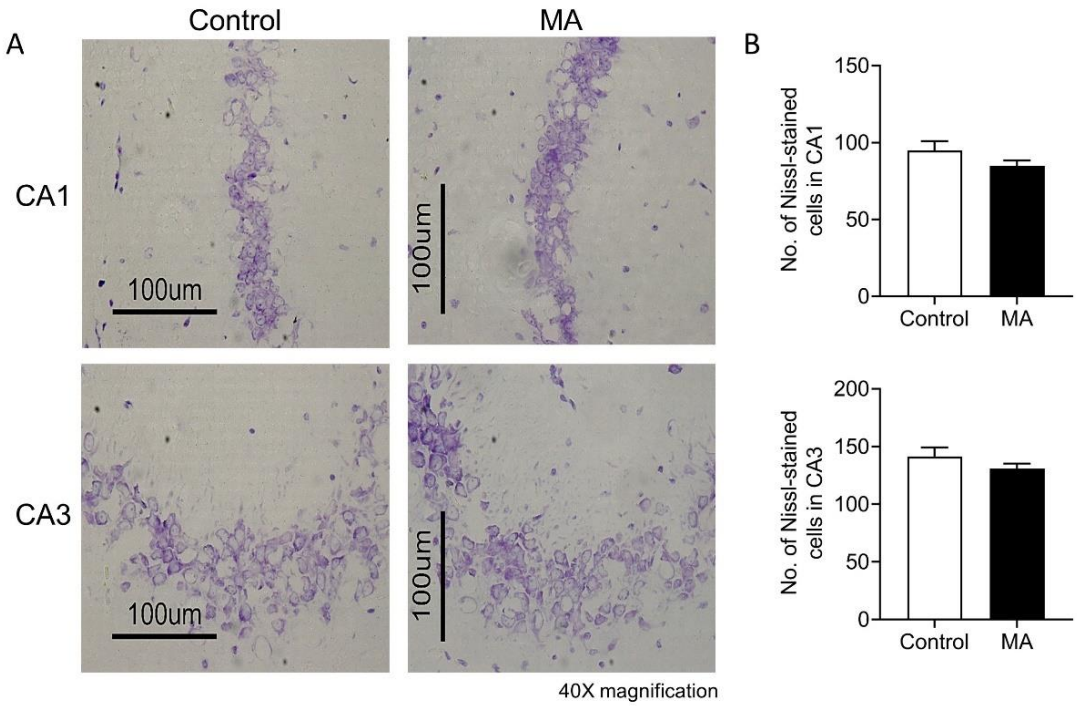


Figure 29 The number of neurons in the hippocampal CA1 and CA3 of adult male mice (12 weeks old)

(A) Nissl-stained neuron. The microscopic image was displayed in 40X magnification.

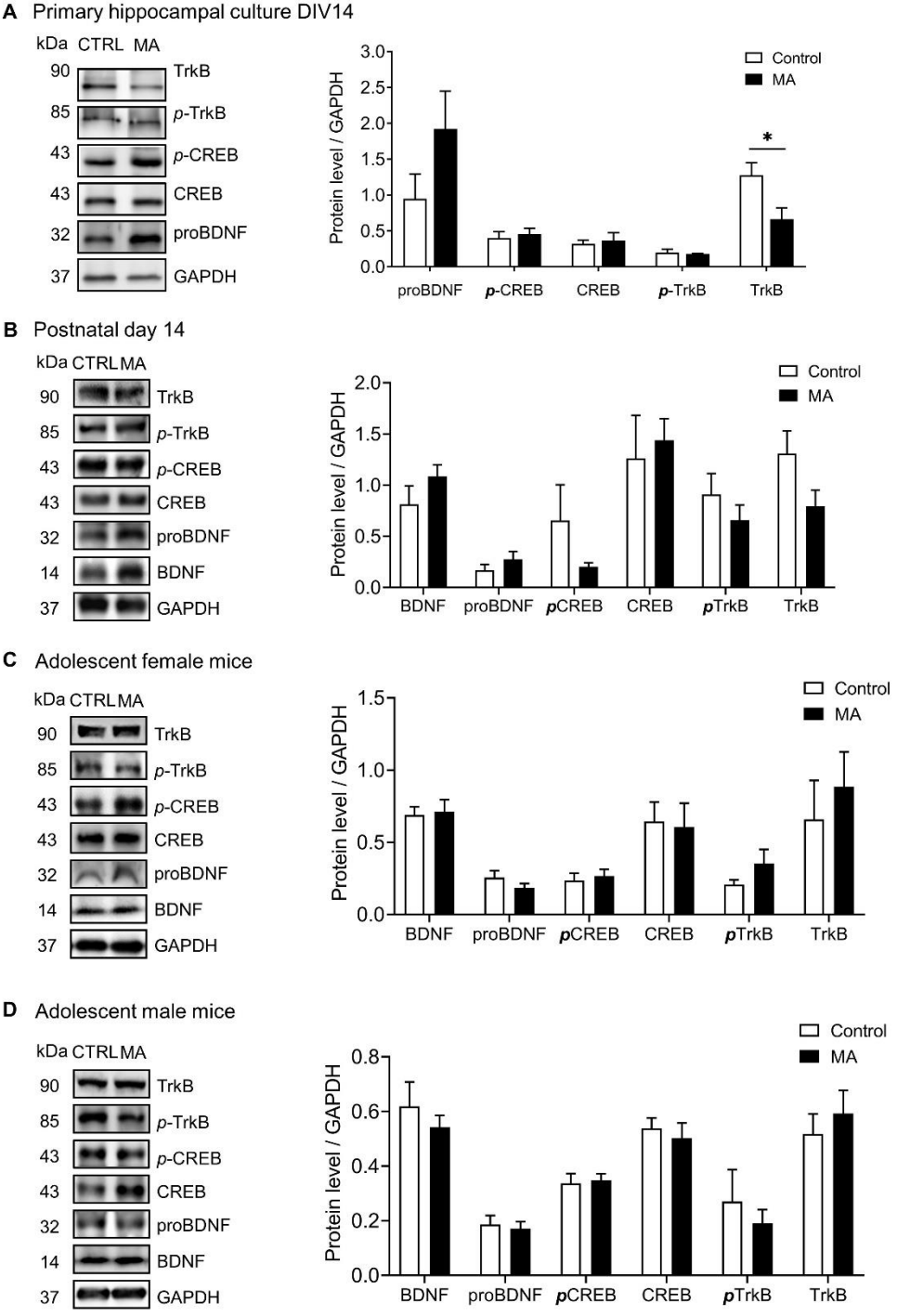
(B) The number of nissl-stained cells was presented as the mean ± S.E.M. (n = 6/group).

4.3 Effect of prenatal MA exposure on BDNF associated protein expression

4.3.1 Protein expression in the hippocampus

In primary hippocampal cultures, proBDNF, *p*-TrkB and TrkB receptor, *p*-CREB and CREB transcription factor expressions were measured using western blot technique. The expression of TrkB receptor was significantly decreased in MA-exposed hippocampal neurons ($t(6) = 2.611$, $p = 0.0401$), while an active form *p*-TrkB was not different between MA-exposed and the control group. BDNF cannot be detected in the primary hippocampal neurons. MA exposure tended to increase proBDNF protein expressions, but this effect was not significantly different from the control mice. In addition, MA exposure did not significantly affect the levels of CREB and *p*-CREB proteins (Fig. 30A)

The expression levels of BDNF associated proteins; proBDNF, BDNF, TrkB, *p*-TrkB, CREB and *p*-CREB were also determined in the hippocampus of infantile, adolescent and adult mice. In PND14 mice, while the expressions of BDNF in the hippocampus tended to increase in MA-exposed mice, this effect was not significantly different from the control mice (Fig. 30B). Other BDNF-related proteins were not affected by MA exposure in every group of adolescent and adult mice (Fig. 30C-D).


A Primary hippocampal culture DIV14
kDa CTRL MA
90 TrkB
85 p-TrkB
43 p-CREB
43 CREB
32 proBDNF
37 GAPDH
Protein level / GAPDH
Control
MA
*
proBDNF p-CREB CREB p-TrkB TrkB
B Postnatal day 14
kDa CTRL MA
90 TrkB
85 p-TrkB
43 p-CREB
43 CREB
32 proBDNF
14 BDNF
37 GAPDH
BDNF proBDNF pCREB CREB pTrkB TrkB
C Adolescent female mice
D Adolescent male mice

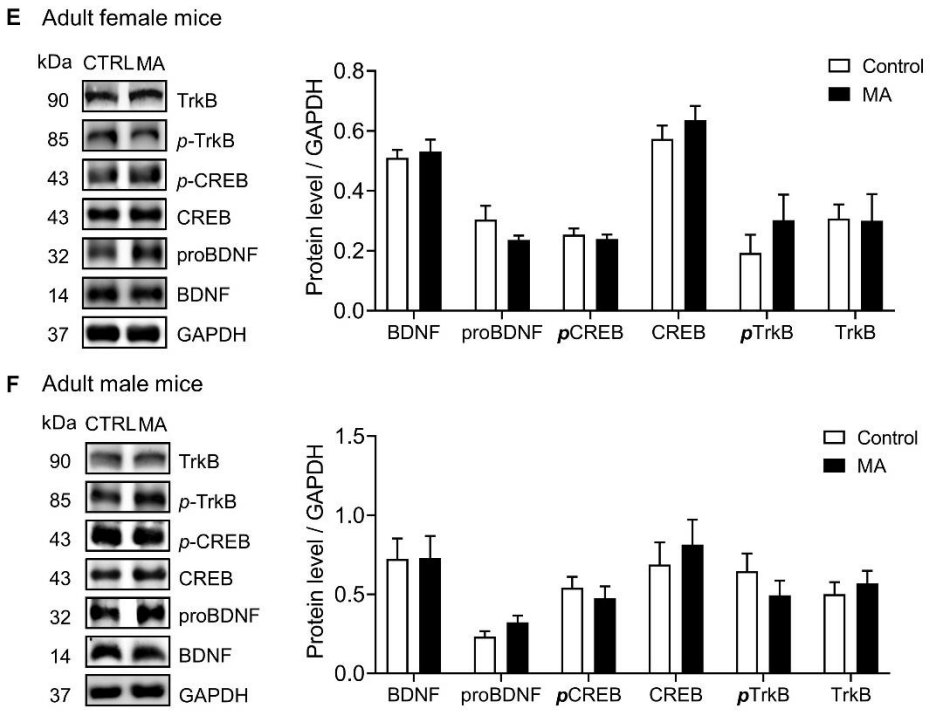


Figure 30 The expression of BDNF-TrkB signaling proteins in the hippocampus Western blot analysis of the primary hippocampal culture at DIV14 (A), hippocampal tissue from postnatal day 14 mice (B), adolescent female (C) and adolescent male mice (D), adult female (E) and adult male mice (F). The band intensity was quantified and normalized to GAPDH. The data are illustrated as the mean ± S.E.M. (n = 4-6/group)

4.3.2 Protein expression in the prefrontal cortex

In PND14 mice, prenatal MA exposure significantly increased the BDNF expression levels in the prefrontal cortex (Fig. 31A; $t(10) = 2.3$, $p = 0.0443$). Regardless of higher BDNF substrate in MA-exposed mice, the expression levels of *p*-TrkB were lower than the control mice in the prefrontal cortex, but these effects were not significantly different. Furthermore, the expression levels of proBDNF, TrkB, CREB and *p*-CREB were not affected by MA exposure.

In adolescent female mice, the expression levels of protein in BDNF-TrkB signaling did not alter in MA-exposed mice compared to the control group (Fig. 31B). However, male adolescent mice showed significantly increased *p*-CREB levels in the prefrontal cortex (Fig. 31B; $t(9) = 3.195$, $p = 0.0109$). Other related proteins did not change in the prefrontal cortex of female and male mice.

MA exposure had no effect on the expression levels of proteins in BDNF-TrkB signaling in the prefrontal cortex of the adult female mice (Fig. 31C). On the other hand, the reduction in *p*-TrkB expression levels was detected in the prefrontal cortex of adult male mice prenatally exposed to MA (Fig. 31C; $t(9) = 2.966$, $p = 0.0158$).

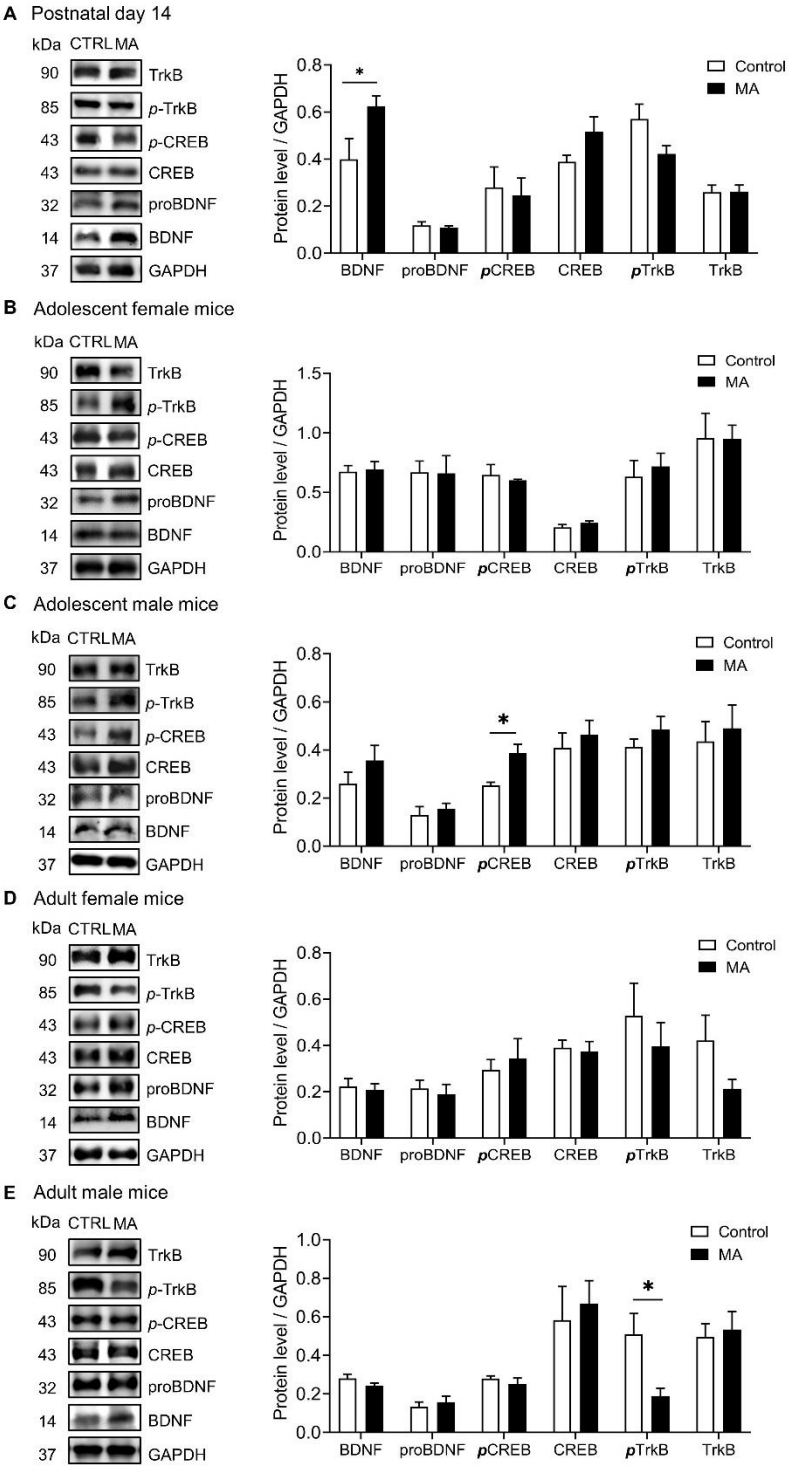


Figure 31 The expression of BDNF-TrkB signaling proteins in the prefrontal cortex Western blot analysis of the prefrontal cortex tissue from postnatal day 14 mice (A), adolescent female (B) and adolescent male mice (C), adult female (D) and adult male mice (E). The band intensity was quantified and normalized to GAPDH. The data are illustrated as the mean ± S.E.M. *$p < 0.05$ vs control group. (n = 6/group)

4.3.3 Protein expression in the striatum

In PND14 mice, prenatal MA exposure significantly increased BDNF expression levels in the striatum (Fig. 32A; $t(8) = 3.61$, $p = 0.0068$). The expression levels of BDNF-related proteins did not change in adolescent and adult mice (Fig. 32B-C). These outcomes were similar to that of the hippocampal tissues, indicating that the striatum of both female and male mice was not permanently affected by prenatal MA exposure.

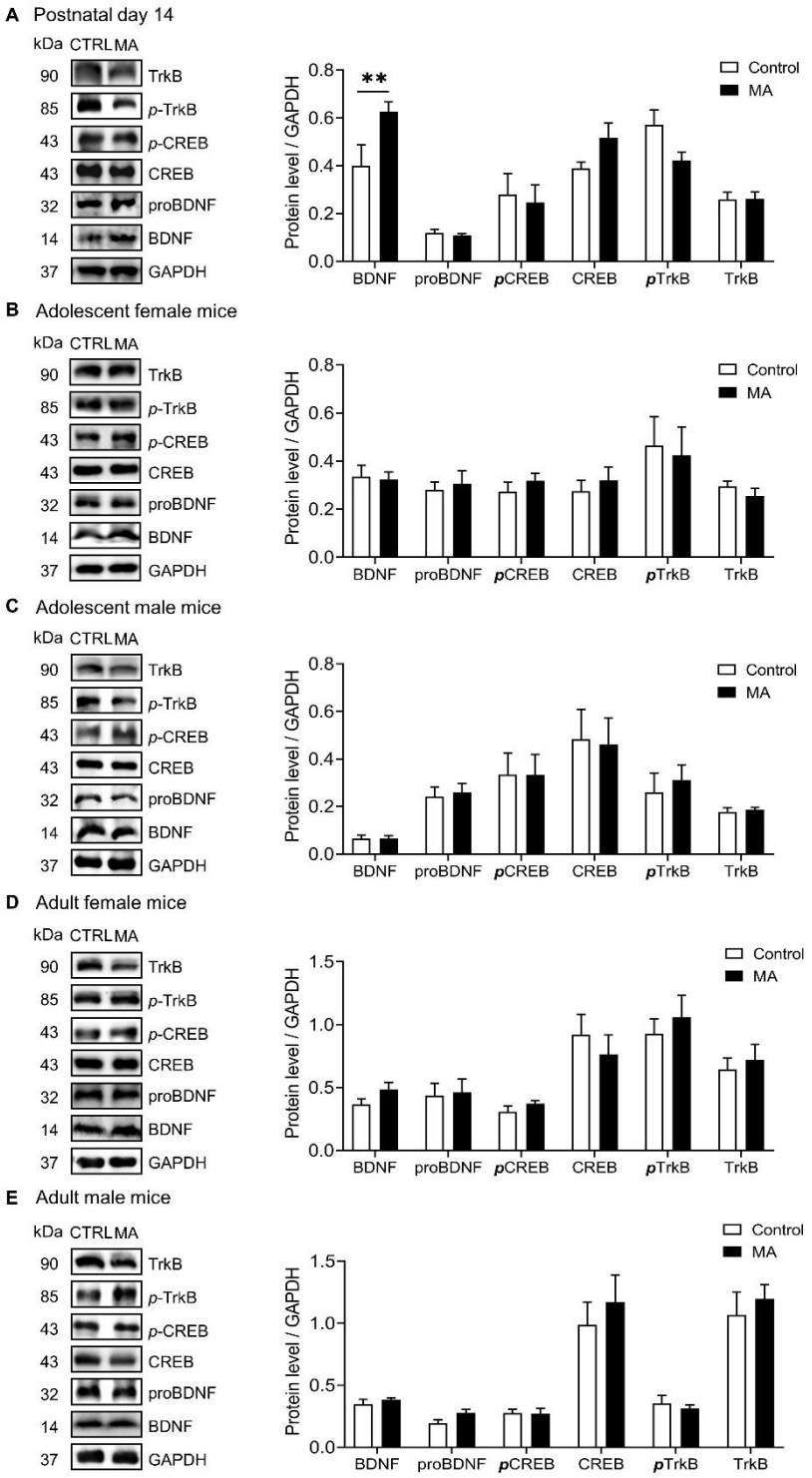


Figure 32 The expression of BDNF-TrkB signaling proteins in the striatum

Western blot analysis of striatal tissue from postnatal day 14 mice (A), adolescent female (B) and adolescent male mice (C), adult female (D) and adult male mice (E). The band intensity was quantified and normalized to GAPDH. The data are illustrated as the mean ± S.E.M. $^{**}p < 0.01$ vs control group. (n = 6/group)

Taken together, BDNF expression was clearly affected by MA exposure in an early stage of life. In infantile mouse with MA exposure, the levels of BDNF were significantly enhanced in the prefrontal cortex and striatum. It also tended to increase in hippocampal tissue and primary hippocampal cultures. Moreover, the results from adolescent and adult mice suggested that male mice were more vulnerable to the effects of prenatal MA exposure than female mice in terms of changes in the BDNF-TrkB protein expression levels. This incident was preferably emerged in the prefrontal cortex in both ages.

CHAPTER V
DISCUSSION & CONCLUSION

The main purpose of this present study was to investigate the effect of prenatal methamphetamine exposure on learning and memory function and synaptic formation, and to elucidate its mechanism of toxicity focusing on BDNF-TrkB pathway. Working memory and spatial memory impairments were observed in the current study with the impact of sex and age. The *in vitro* study in primary hippocampal culture of MA-exposed embryo demonstrated the detrimental effects on neurite growth and synaptogenesis. The presynapse was significantly declined in embryo' s hippocampal culture and in PND14 mice, while the alteration of postsynaptic protein levels was noted in the hippocampus of adolescent and adult mice. The effects of prenatal MA exposure on BDNF expression and TrkB receptor were detected specifically in the prefrontal cortex, but not in the hippocampus. Therefore, the current study suggested that neuronal structure and synaptic communication might be diminished resulting in the impaired learning and memory in prenatal MA-exposed mice.

5.1 Effects of MA on matenal weight and effects of *in utero* MA exposure on embryo and offspring physical appearance

MA possesses an anorectic effect (Cruickshank & Dyer 2009) which might affect maternal weight. However, in this study, there was no difference in maternal weight gain throughout MA administration period. Moreover, the dose of MA used in this study (5 mg/kg) showed no fatal effect on pregnant mice and offspring. Other studies also observed the similar trend of constant maternal body weight after receiving MA at the dose of 5 mg/kg (Pometlová et al 2009, Šlamberová et al 2005). This finding can exclude an influence of nutrient deficiency on any outcomes in this study.

The teratogenic effect of MA was reported in previous studies with dose-dependent manner and gestation-specific (Gomes et al 2012, Mirjalili et al 2013,

Yamamoto et al 1992). In this study, MA-exposed embryos had a significantly small head size, but changes in crown-lump length was not observed. This finding was in consistent with previous reports in human and animals showing that MA treatment during pregnancy resulted in delayed development of early growth (Abar et al 2014, Acuff-Smith et al 1996a, Smith et al 2003). The alteration of phenotypic features can result from the direct effect of MA toxicity and the changes of maternal conditions such as energy metabolism (Dicke 1989, Graf et al 2013). Moreover, the sympathomimetic effect of MA causes high blood pressure and vasoconstriction (Rommel et al 2016) leading to low utero-placental blood supply, hypoxic condition in fetus, and oxidative stress (Ross et al 2015). A hypoxia model in pregnant mice demonstrated the fetal growth restriction (Kusinski et al 2012). In addition, Well et al. (2016) proposed that the activations of NADPH oxidases (NOX) and prostaglandin H synthases (PHS) by prenatal MA treatment mediated ROS formation in fetal brain, which might be responsible for neurodevelopmental deficits (Wells et al 2016). The physical characteristics were continuously recorded in postnatal day14, 6-week-old and 12-week-old mice. The data showed no difference between groups, except female adult mice with significantly smaller skull in MA-exposed group. This observation is in line with previous studies that displayed a delay in somatic development only in neonatal mice but no deficit in body weight and length after that (McDonnell-Dowling et al 2014). It can conclude that MA is likely to delay a developmental process of skull in an early phase, but there is no long-lasting effect into postnatal period.

5.2 Effects of prenatal MA exposure on behavioral functions

Behavior test was conducted in 6-week-old and 12-week-old mice. The results showed normal locomotor activity in MA-exposed mice which is in consistent with previous study (Sato and Fujiwara 1986). In addition, previous studies also showed no change in dopamine levels of prenatal MA-exposed mice and rats (Sato and Fujiwara 1986, Won et al 2002). Thus, it is hypothesized that dopamine levels in prenatal MA-exposed mice were also unaltered in the present study. The locomotor

activity result could assure a normal stage of exploratory behavior and movement, so there was no interaction with other behavioral tests.

The effects of prenatal MA exposure in adolescent mice on memory were different in males and females. MA-exposed male mice had working memory impairment, whereas spatial-related memory impairment was presented in MA-exposed female mice. This inconsistency could be supported by previous studies which identified the effect of gender difference in behavioral test, particularly learning and memory models. Accumulating evidence suggested the differences in exploration strategy to novel recognition. To recognize the objects, female mice used local cues such as special features of the objects, while male mice preferred to use distal cues with directional information (Bettis & Jacobs 2012, Frick & Gresack 2003). Thus, female mice could pay more attention and acquire more information in identifying the objects (Bettis & Jacobs 2009), leading to the higher performance in the NOR test. On the other hand, male advantage was demonstrated in spatial and cued-navigation task (Berger-Sweeney et al 1995). Extensive studies confirmed that male mice preferentially utilized visible landmarks for orientation than did females (Saucier et al 2008, Voyer et al 2007). Furthermore, these different performances could relate to the stage of estrous cycle in female rodents. The levels of ovarian steroids, particularly estrogen and progesterone affected cognitive performance by regulating electrophysiological property and synapse plasticity (Smith et al 2002, Woolley & McEwen 1992). Many researches suggested that elevated estrogen levels in pro-estrous phase played an inhibitory role in spatial- or hippocampus- related memory (Galea et al 2001, Stackman et al 1997, Sutcliffe et al 2007). Despite controversy effect of estrogen on working memory, some studies revealed a beneficial effect of estrogen during pro-estrous phase in NOR model over other estrous stages (Walf et al 2006). The finding of this study offered supporting evidence that MA exposure during brain development can induce a memory deficit in adolescence. This impairment was influenced by sex that emerged as defective working memory in male and impaired spatial memory in female adolescent offspring.

The present study showed that prenatal MA treatment revealed the long-lasting negative impact on recognition and spatial memory in adult mice without sex bias. Normally, memory impairment is observed in aged mice (18-25 months old) (Benice et al 2006, Frick et al 1999). This study demonstrated that prenatally exposed to MA can cause memory deficits in young adult mice (12 weeks old). Previous studies revealed the conflicting results of effect of prenatal MA exposure on learning and memory in adults. Some studies showed no impact of low dose MA exposure throughout the gestation period on learning and memory performance in Morris water maze (MWM) (Jalayeri-Darbandi et al 2018, Macúchová et al 2014), while other studies showed that MA treatment during GD 12-22 induced better performance in memory recall (Hrebíčková et al 2016, Schutová et al 2009). In contrast, prenatal MA exposure produced the impairments in various memory types; for example, MWM recall memory in female (Macúchová et al 2013b), MWM new learning ability in high dose treatment (Acuff-Smith et al 1996b) and NOR visual recognition memory in male (Dong et al 2018). In addition, postnatal exposure to MA had a significant effect on recognition memory (Siegel et al 2010) and MWM recall memory (Vorhees et al 2000, Williams et al 2003). The preceding evidence indicated a long-lasting effect of MA in every stage of developing brain that mimics trimester of pregnancy in human including first and second half of mouse gestation day, and neonatal period. This study supported a negative effect of prenatal MA exposure during second trimester on spatial-related memory and short-term recognition memory.

Adult uses of MA have been generally recognized its toxicity on memories in both human abusers and animal models (Bigdeli et al 2015, Melo et al 2012, Scott et al 2007). Cognitive function permanently disrupted by MA in adult mainly results from monoamine neurotransmitter-related alteration, especially dopamine-induced neurotoxicity (Kokoshka et al 2000, Schaefer et al 2008). Although a similar pattern of memory impairment was also found in mice exposed to MA during pregnancy, the neurochemical and structural changes in brain function of prenatal MA-exposed mice have not been elucidated. However, it is hypothesized that the neurotoxic effects are not related to dopamine.

Anxiety is often reported as the adverse effects in MA abusers (Zweben et al 2004), while its effect in animal models remains unclear. Previous study reported that anxiogenic-like behavior was presented immediately after MA treatment, but anxiolytic-like behavior was found as a delayed effect (Miladi-Gorji et al 2015). In contrast, a study by Macúchová et al. (2016) demonstrated the anxiolytic-like effect of acute MA treatment (Macúchová et al 2016). In this study adolescent and adult mice from MA group did not show anxiety-like behavior. The result is in agreement reporting no effect of *in utero* and postnatally exposed to MA via breast milk on anxiety behavior in mice (Hrubá et al 2012). The absence of anxiety-related behaviors in this study ensured that exploratory behavior in the memory test was not confounded by anxiety-related effect.

Depression is a common comorbid immensely found among MA addicts (Glasner-Edwards et al 2009). Several animal studies demonstrated that MA administration can induce a long-lasting depressive-like symptom, as well as after withdrawal stage (Mouton et al 2016, Shabani et al 2019). The present study also evaluated prenatal MA effect on depressive-like behavior and reported no difference between MA and the control group in both ages. Serotonergic system plays a major role in depression (Morrissette & Stahl 2014). There was a discrepancy among previous studies of prenatal effect of MA on the serotonin system in offspring. Low dose of prenatal MA (2 mg/ kg) treatment leaded to a decrease in 5-HT receptor density in the frontal cortex, and a reduction of 5-HT reuptake transporter in many parts of the brain (Sato & Fujiwara 1986, Weissman & Caldecott-Hazard 1993), whereas treatment of MA 5 mg/kg at late gestation stage did not alter the density of cortical 5-HT terminals and 5-HT receptors (Cabrera et al 1993). On the other hand, 5 mg/ kg MA treatment throughout gestational day showed an increase in basal concentration of serotonin in adult mice (Fujáková-Lipski et al 2017). High dose of MA (10-40 mg/kg) injection during pregnancy caused an opposite effect as increment of 5-HT uptake sites and an elevation of 5-HT levels (Weissman & Caldecott-Hazard 1993, Won et al 2002). The variation of prenatal effects could be contributed by dose, stage of exposed gestation and brain regions. Our result here supported the

accompanying effect on behavior that MA did not have lasting effect on depression-related behavior of the progeny.

5.3 Effects of prenatal MA exposure on neuronal morphology and synapse

Since neural circuits within the hippocampus highly contribute to memory processes, the present study assessed the number of neurons in the hippocampal CA1 and CA3 areas. There is strong evidence supported the neurotoxicity of MA on neurons in many brain regions (Cadet et al 2005), but long-term effect following maternal MA administration is still unclear. Previous research has been hypothesized that cognitive impairment might be partially caused by MA-induced neuronal death (Jablonski et al 2016). Some researchers also proposed that the prenatal MA exposure had a potential neurotoxicity on developing brain via endoplasmic reticulum (ER) and oxidative stress-induced neuronal damage (Tsai et al 2019). Earlier *in vitro* studies exhibited a reduction of hippocampal neural progenitor cells (NPCs) proliferation by MA, leading to less newly generated adult hippocampal neurons (Venkatesan et al 2011). It is noted that neurotoxicity of MA in this *in vitro* study was exerted in much higher concentration of MA treatment comparing to those found in pup's brain following 5 mg/kg MA maternal injection (Rambousek et al 2014). MA treatment also interrupted dentate gyrus (DG) self-renewal capacity and cell cycle (Baptista et al 2014). However, the present study observed no long-lasting effect of MA exposure on neuronal number in the hippocampus. This could be due to a plasticity property of the hippocampus that cell death and newly production has been ongoing throughout life (Leuner & Gould 2010). This finding could be supported by the study from Bagheri J and colleagues showed that neuronal apoptosis in DG, CA1 and CA3 region of the hippocampus was observed only at PND 1 after prenatal MA treatment throughout gestation day, but lasting effect was not found in juvenile mice (Bagheri et al 2017). Due to morphologically and functionally immature of early postnatal developing brain, an adaptation might be applied during the immense neurogenesis period. Based on various results, it could be convinced that the development of hippocampal neurons of prenatally MA-exposed mice is still intact

in later life. Other depletion of hippocampal functions should attribute to MA-induced memory impairment rather than cell death.

In this study, the hippocampal neuron morphology of mouse embryo was examined using primary culture technique. The reductions of dendrite ramification, axon length and diameter were observed in the hippocampal primary cultures from MA-exposed mice. Previous study also demonstrated a decrease in dendritic spine density in the hippocampus as a continual effect of MA treatment in early postnatal life (Williams et al 2004). In addition, the reduced axonal fiber size of optic nerves was also reported in prenatally MA-exposed mice (Melo et al 2008a). Changes in dendritic and axonal morphology are associated with the decreased microtubule associated protein 2 (MAP2) gene expression, which is a cytoskeletal protein in dendrite involving in neurite outgrowth and neuronal plasticity (Putzke et al 2007), and with the reduction of neural cell adhesion molecules (NCAMs) in the hippocampus (Baei et al 2017). Therefore, prenatal MA-induced cytoskeletal protein reductions lead to deficient neuronal growth, which might evoke cognitive dysfunctions in later life.

Even though the number of neurons in the hippocampus remained stable until adulthood, the hippocampal synapses were affected by prenatal MA treatment. This study was for the first time demonstrating prenatal effect of MA on developing neurons at the critical period of synaptogenesis in perinatal day. The results from MA-exposed hippocampal primary cultures depicted a significantly smaller area of colocalization between pre- and postsynapse. The synapsin-stained nerve terminal was also substantially decreased in hippocampal cultures from MA-exposed embryo. This finding was consistent with diminishing synapsin-1 expressions, a presynaptic protein in 14-day-old mice after prenatally being exposed to MA. Synapsin-1 is a synaptic vesicle-anchoring protein that plays the critical role in regulating neurotransmitter release, synapse formation and axon elongation (Cesca et al 2010). Cytoskeletal-mediated axonal transport was necessary to transfer various synaptic components to nerve terminals, in favor of maintaining synapse connectivity (Chowdary et al 2012). MA-induced axonal and presynaptic loss reported in this study could lead to a deteriorative synaptic transmission and cognitive decline. Prenatal

MA-induced presynaptic protrusion might be a consequence of the defective axons observed in the present study.

In contrast to the results in the primary hippocampal cultures and PND14 brains, the expression levels of postsynaptic protein, PSD-95, were escalated by MA in adolescent and adult mice, while the expression levels of presynaptic protein, synapsin-1, remained constant. Only female adult exhibited a lower expression of synapsin-1 as a result of prenatal MA treatment. The negative effect was highly pronounced in response to prior treatment might be resulted from sex-different modifications at baseline. Stress-induced structural plasticity pattern in the hippocampus was different between sex, such as less dendritic branching in female (Galea et al 1997). Apart from an obvious fluctuation of hormone through estrogen receptor, many mechanisms were suggested to mediate this difference including NMDA receptor signaling, opioid receptor and nitric oxide level (Hyer et al 2018). An increase in PSD95, a postsynaptic marker in excitatory synapse, presumably resulted from homeostatic plasticity to overcome a presynaptic change in transmission (Turrigiano & Nelson 2004). Homeostasis compensation was implemented through various of mechanisms that were engaged to stabilize an appropriate range of neural network functions, including regulation of postsynaptic receptor or neurotransmitter release (Harris & Littleton 2015). Furthermore, there was a study revealing an inverse correlation between PSD-95 expression and drug-related plasticity that a downregulation of PSD-95 can enhance long-term potentiation (LTP) (Yao et al 2004). Thereby, an elevated expression of PSD95 shown in this study is proposed to compensate the presynaptic loss to maintain memory function.

Accumulating evidence showed that the morphology of dendrites and axons was modified by MA treatment during gestation. Likewise, prenatal MA exposure could exert a negative effect on synaptic organization without the neuronal degeneration. The molecular mechanisms could partially be influenced by activity-dependent changes in synaptic plasticity. Nonetheless, an impairment of memory performance was long-lasting expressed in later life. A subtle change in synapse organization or morphology resulted in impaired memory performance (Corradi et al 2008, Rosenberg et al 2014). Overall, these findings suggest that the alterations of

neuronal synapse in the hippocampus contribute to the neurocognitive impairments in this study.

5.4 Effects of prenatal MA exposure on associated proteins in BDNF-TrkB pathway

In the primary hippocampal cultures, an expression of protein involved in BDNF-TrkB pathway was evaluated to determine the mechanism mediated the malformation of developing neurite and synapse morphology. BDNF is a crucial protein in the neurotrophin family that regulates many aspects of neural circuit development and activities, including neuron proliferation, neurite growth, synaptogenesis and synaptic plasticity (Reichardt 2006). BDNF is initially produced in precursor form, proBDNF, which is secreted and cleaved to mature BDNF, mostly by extracellular protease (Teng et al 2005). In this study, intracellular proBDNF but not BDNF was detected in the primary hippocampal neurons. Prenatal MA administration tended to enhance proBDNF expression in the hippocampal culture. TrkB, BDNF-specified receptor, was significantly downregulated by prenatal MA, but *p*-TrkB expression levels were unaltered. Previous studies suggested that excitotoxic stimulation leaded to TrkB receptor degradation via calpain activation (Gomes et al 2012, Jerónimo-Santos et al 2015). Calpains are the Ca^{2+}-dependent protease family that play many pivotal roles in neurological functions. MA activated an increase in intracellular Ca^{2+} through the dopaminergic and glutaminergic systems (Kim et al 2020). It is hypothesized that prenatal MA-induced excitotoxicity in the brain induced calpain activity to degrade TrkB expression. In addition to the Trk family, proBDNF can bind with higher affinity to p75 neurotrophin receptor ($p75^{NTR}$). This signaling mostly leads to apoptotic pathway and growth retraction (Meeker & Williams 2015). Since an expression of *p*-TrkB remained constant, it is hypothesized that proBDNF, detected in this study, activates $p75^{NTR}$, but not TrkB, resulting in the deteriorating effect of MA in the primary hippocampal neurons.

The levels of BDNF were significantly increased in the prefrontal cortex and striatum of PND14 mice prenatally exposed to MA. However, no change of BDNF levels was observed in the adolescent and adult mice. A result is in agreement with

previous studies giving MA to neonatal mice. It was shown that MA treatment in neonates increased the BDNF expressions in the hippocampus and striatum only after treatment (Grace et al 2008), but BDNF levels then returned to normal in later life (Skelton et al 2007). Similar pattern was found in MA-administered to adult rodents. BDNF protein levels could be augmented promptly after treatment, but then the level was decreased as long-term effect (Galinato et al 2015, Krasnova et al 2013). Taken together, even though the fluctuation of BDNF expressions was evidenced in many studies including the present study, it is hypothesized that the alteration of BDNF levels in early life could affect MA-induced memory deficit in adolescence and adult.

The alterations of protein expressions in the BDNF-TrkB pathway were detected in the striatum and prefrontal cortex of prenatally MA-exposed mice but not in the hippocampus. Since dopaminergic neurons in the striatum is the primary target of MA toxicity, it is postulated that MA could affect the neuroadaptation signaling in the striatum. Previous study revealed that an excitotoxic lesion in the striatum could promote BDNF transcription and expression (Rite et al 2003). Moreover, the modification of BDNF and dopamine receptor expressions could be detected in early stage following MA treatment, not after 6 weeks. (Thompson et al 2015). In the same way, this study demonstrated the increase in BDNF protein expressions in the striatum of PND14 mice exposed to MA prenatally, suggesting the neuroadaptation in the striatum due to MA neurotoxicity.

Interestingly, BDNF-TrkB pathway in the prefrontal cortex was mostly affected by prenatal MA exposure, especially in male mice. The prefrontal cortex plays a critical role in learning and memory, especially working memory. The hippocampus-prefrontal pathway through the direct and indirect dopaminergic and glutamatergic projections is activated during memory task (Thierry et al 2000). The glutamatergic system plays an important role in learning and memory processes, neuronal growth and synaptic plasticity (Riedel et al 2003). Thus, a reduction of frontal activity might be, in part, responsible for memory impairment and activity-dependent plasticity. Previous studies also reported hypometabolism and frontal executive dysfunction in MA users (Kim et al 2009), specifically more severe in male users (Kim et al 2005).

The metabolomic study in rodent revealed that MA disrupted energy metabolism and glutamatergic neurotransmission in the hippocampus and prefrontal cortex together with mitochondrial toxicity and oxidative stress (Bu et al 2013, McClay et al 2013, Shima et al 2011, Zheng et al 2014). Collectively, MA-induced hypofrontality can be implied from these data indicating reciprocal role of the prefrontal cortex in memory deficit through the glutamatergic circuitry. Further analysis on glutamatergic neuron loss in the prefrontal cortex, and its receptors including NMDA subtype in the hippocampus should be conducted to confirm this association.

BDNF and related proteins did not change in the hippocampus, indicating that this pathway did not account for synaptic plasticity found in the hippocampus of adolescent and adult mice. Apart from BDNF-TrkB pathway, exposing to stress during pregnancy can interfere hippocampal adult neurogenesis and morphological changes through stress hormones (Ortega-Martínez 2015). Prenatally MA-exposed rats exhibited an upregulation of glucocorticoid receptor gene expression in the brain and elevated corticosteroid release (Zoubkova et al 2019, Zuloaga et al 2015). Previous study revealed the different effect of glucocorticoid receptor (GR) and mineralocorticoid receptor (MR). While GR activation resulted in the suppression of morphological development of neurons, MR showed the opposite effect on hippocampal cultures (Gould et al 1999). Prenatal stress decreased MR expression leading to a malfunction of morphology and functional maturation of hippocampal neurons later in life (Tamura et al 2011). Given the controversial results from prenatal MA treatment regarding receptor distribution, this pathway should be further investigated to assure mechanism mediated MA-induced morphological and synaptic alteration in hippocampus.

5.5 Conclusion

Overall, prenatal MA exposure induced learning and memory impairment in adolescent and adult mice, which resulted from the disruptions of hippocampal neuronal growth and synaptogenesis in the developing brain. The effects on the BDNF-TrkB signaling pathway were exclusively found in early age. There were ongoing changes in postsynaptic proteins as a neuroadaptation. Furthermore, the prefrontal

cortex was largely affected by prenatal MA exposure that associated with an impairment of memory function.

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90

APPENDIX

Animal Use Certificate

Chulalongkorn University Animal Care and Use Committee

Certificate of Project Approval	☑ **Original** ☐ **Renew**
Animal Use Protocol No. 16-33-007	**Approval No. 16-33-007**

Protocol Title
Effect of prenatal methamphetamine exposure on synaptogenesis in hippocampal primary cell culture

Principal Investigator

Ratchanee RODSIRI, PhD

Certification of Institutional Animal Care and Use Committee (IACUC)

This project has been reviewed and approved by the IACUC in accordance with university regulations and policies governing the care and use of laboratory animals. The review has followed guidelines documented in Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes edited by the National Research Council of Thailand.

Date of Approval	**Date of Expiration**
October 18, 2016	October 17, 2018

Applicant Faculty/Institution
Faculty of Pharmaceutical Sciences, Chulalongkorn University, Phyathai Road., Pathumwan BKK-THAILAND. 10330

Signature of Chairperson	**Signature of Authorized Official**
Name and Title **THONGCHAI SOOKSAWATE, Ph.D.** **Chairman**	Name and Title PORNCHAI ROJSITTHISAK, **Ph.D.** **Associate Dean (Research and Academic Service)**

The official signing above certifies that the information provided on this form is correct. The institution assumes that investigators will take responsibility, and follow university regulations and policies for the care and use of animals.

This approval is subjected to assurance given in the animal use protocol and may be required for future investigations and reviews.

Chulalongkorn University Animal Care and Use Committee

Certificate of Project Approval	☑ Original ☐ Renew
Animal Use Protocol No. 18-33-013	**Approval No. 18-33-013**

Protocol Title
Effect of prenatal methamphetamine exposure on neurobehavioral function

Principal Investigator

Ratchanee RODSIRI, PhD

Certification of Institutional Animal Care and Use Committee (IACUC)

This project has been reviewed and approved by the IACUC in accordance with university regulations and policies governing the care and use of laboratory animals. The review has followed guidelines documented in Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes edited by the National Research Council of Thailand.

Date of Approval	**Date of Expiration**
September 28, 2018	July 31, 2020

Applicant Faculty/Institution
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Signature of Chairperson	**Signature of Authorized Official**
Name and Title **THONGCHAI SOOKSAWATE, Ph.D.** **Chairman**	Name and Title **PORNCHAI ROJSITTHISAK, Ph.D.** **Associate Dean (Research and Academic Service)**

The official signing above certifies that the information provided on this form is correct. The institution assumes that investigators will take responsibility, and follow university regulations and policies for the care and use of animals.

This approval is subjected to assurance given in the animal use protocol and may be required for future investigations and reviews.

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PUBLICATION	Teerapattarakan N, Benya-Aphikul H, Tansawat R, Wanakhachornkrai O, Tantisira MH, Rodsiri R. Neuroprotective effect of a standardized extract of Centella asiatica ECa233 in rotenone-induced parkinsonism rats. Phytomedicine. 2018. 15;44:65-73. Boonlert W, Benya-Aphikul H, Umka Welbat J, Rodsiri R. Ginseng Extract G115 Attenuates Ethanol-Induced Depression in Mice by Increasing Brain BDNF Levels. Nutrients. 2017. 24;9(9).
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