CHAPTER III

RESEARCH METHODOLOGY

Animals

Adult male Sprague-Dawley rats (National Laboratory Animal Center, Mahidol University, Nakorn Pathom, Thailand) weigh between 200-250 g were used in this study. The animals were housed one per cage (30x19x13 cm) and maintained at room temperature about 24±1°C under light dark cycle 12:12, light on at 6:00 A.M., with *ad libitum* access to food and water. Before drug treatment, all animals were habituated for 5 days. All animal procedures were carried out in compliance with Mahidol University code of practice and the National Institutes of Health (USA) Guidelines for treatment of laboratory animals. This research has been approved by the Animal Research Committee of Naresuan University, Thailand.

General procedure

Before drug treatment of each day, all animals were weighed to calculate amount of drug which used to prepare drug. Food intakes were weighed to measure the amount of food that was obtained. Core temperature was rectally measured at 6:00 A.M. and 1 h after the last dose injection on day 0-13 and 1 h after METH binge on day 15 to avoid hyperthermia.

Drug

D-methamphetamine hydrochloride (Lipomed AG, Arlesheim, Switzerland) with the permission of Ministry of Public Health were used in this experiment. The drug was dissolved in saline (0.9% w/v of NaCl) and administered via intraperitoneal injection (2 ml/kg). Drug was made up fresh each day and all doses drug injection volume based on body weight of animals were administered.

Drug treatment

Drug doses for treatment of the animals were used as followed by Segal, et al. (2003). Animals were divided into four groups: control group (n = 8), acute dose-METH

binge group (AB-METH) (n = 5), escalating dose-METH group (ED-METH) (n = 4) and escalating dose-METH binge group (ED-METH binge) (n = 4). All animals were administered with saline (2 mg/kg) at least one injection per day to familiarly before drug treatment. At the phase of drug administration; control group – rats were intraperitoneally administered with three doses of saline at 3 h intervals on day 1-14 and four doses of saline at 2 h intervals on day 15, respectively; AB-METH group – rats were injected with three doses of saline at 3 h intervals on day 1-14 and four consecutive injections of 6.0 mg/kg at 2 h intervals on day 15, respectively; ED-METH group – rats were injected gradually increasing three doses of METH (0.1-3.9 mg/kg/day) at 3 h intervals on day 1-13 and three doses of 4.0 mg/kg METH at 3 h intervals on day 14 and four doses of saline at 2 h intervals on day 15, respectively; ED-METH binge group – rats were injected as same as ED-METH group on day 1-14 and four consecutive injections of 6.0 mg/kg METH at 2 h intervals on day 15, respectively. At the last dose of each day, the behaviors of animals were observed using behavioral rating scale and locomotor activity test, while novel object recognition test was observed on day 15-16. Rats were sacrificed and brains were removed at 24 h after the last injection on day 16. Diagrammatic of methamphetamine administration is shown in Figure 13. Drug administration for ED binge dose pretreatment schedule is shown in Table 1.

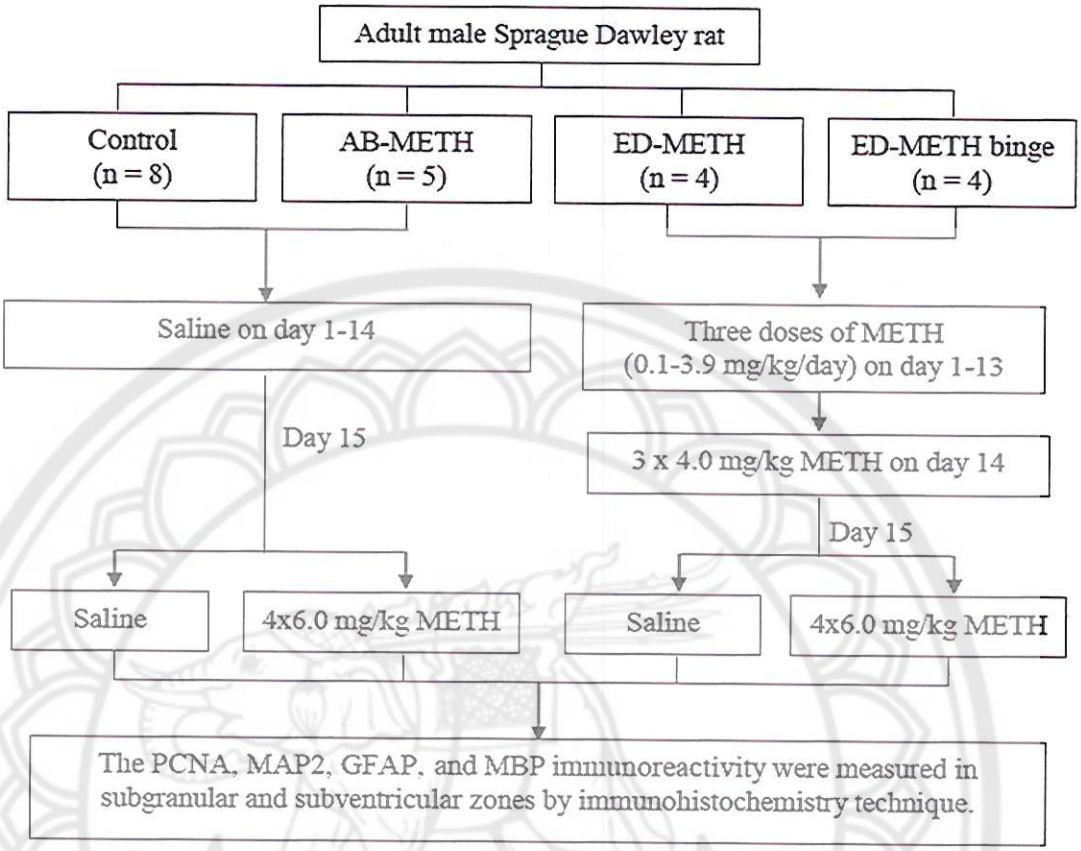

Figure 13 Diagrammatic representations of methamphetamine administration

Table 1 Escalating doses pretreatment methamphetamine schedule

Day	Methamphetamine dose (mg/kg)			
	07:30	10:30	13:30	
1	0.1	0.2	0.3	
2	0.4	0.5	0.6	
3	0.7	0.8	0.9	
4	1.0	1.1	1.2	
5	1.3	1.4	1.5	
6	1.6	1.7	1.8	
7	1.9	2.0	2.1	
8	2.2	2.3	2.4	
9	2.5	2.6	2.7	
10	2.8	2.9	3.0	
11	3.1	3.2	3.3	
12	3.4	3.5	3.6	
13	3.7	3.8	3.9	
14	4.0	4.0	4.0	
Day	**07:30**	**09:30**	**11:30**	**13:30**
15	6.0	6.0	6.0	6.0

Source: Segal, et al., 2003

Behavioral Test

The behavioral test was used their home cage (30x19x13 cm). At the last dose injection of each day, animal behaviors were observed in their home cage for 30 min. Thereafter, rat behaviors were scored using the behavioral rating scale that is modified by Davidson, et al. (2005) (Table 2). The data were collected at the first 20 seconds of each period; 5, 10, 15, 20, 25, 30 min, respectively. During observation, their behaviors were recorded using video record. In this study used the H264 Webcam 3.89 software, which is software of video record was downloaded from http://www.h264soft.com/ free software supplied by Timhillone software.

Table 2 A modified versions of the Ellinwood and Blaster (1974) behavioral rating scale after methamphetamine administration

Score	Classification	Definition
1	Asleep	Lying down, eyes closed
2	Almost asleep	Relaxed muscles, eyes partially shut
3	Dystonia	Abnormal posture, tense muscles
4	Inactive	Lying down, eyes open, infrequent sniffing
5	Grooming	Grooming of face, body or groin
6	Normal active movement	Investigation or sniffing of cage, rearing
7	Hyperactive	Running with rapid jerky positional changes
8	Slow patterned movement	Repetitive exploration
9	Fast patterned movement	Intense, rapid repetitive exploration of cage
10	Stereotypy	In-place sniffing or grooming

Source: Davidson, et al., 2005

Locomotor activity test

The locomotor test is the most common test to evaluate the behavioral effect of drug treatment. After the last dose injection, all animals were tested locomotor activity that adapted from Haider, et al. (2004) and Khaliq, et al. (2008). The locomotor chamber (76x76 cm with walls 42 cm high), used in this experiment, is divided the floor by black lines into 25 equal rectangles (Figure 14). For start experiment, animal were left at center of locomotor chamber. All animals were habituated on locomotor chamber 10 min for 7 days. At testing phase, all animals were placed in the central of locomotor chamber for 10 min. Locomotor activity was scored when animals crossing the black line by both of foot pair. The data were collected at 6-10 min after place animal. Before place each animal, the chamber was cleaned with 10% ethanol.

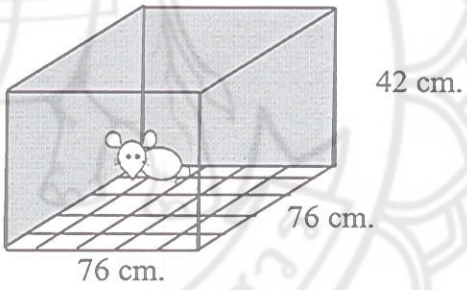

Figure 14 Locomotor chamber (76x76 cm with 42 cm walls high)

The novel object recognition test

The novel object recognition was tested for measuring memory and attention of the animals. The test using in this study was modified from Tuon, et al., 2008; Gomes, et al., 2009. The open field chamber (40x60 cm with 50 cm high walls) with frontal glass wall was used in this experiment. The floor of open field chamber was divided into 12 equal rectangular by black line. At 1 day before experiment, each animal was habituated by leaving in the apparatus without object for 5 min. This test is comprised of training session and testing session. For the training session, animal was left in the chamber with the familiar objects (A1, A2) for 5 min. To testing session, the novel object (B) was replaced to the familiar object (A2) and animal was

left in the chamber for 5 min for exploring the objects. The novel object recognition test is included the short-term, and long-term memory recognition tests. The short-term memory was tested 1.30 h after training session and long-term memory was tested 24 h after training session. In this experiment was record using video recorder as same as behavioral test. The objects were cleaned with 10% ethanol during trial. The data are a duration time that animal was spent for investigating the objects. The data were calculated by following formula.

$$\text{Expression of memory} = t_{novel} / (t_{novel} + t_{sample})$$

t_{novel} = time spent exploring the object A

t_{sample} = time spent exploring the object B

The open field and their objects which are used in this experiment were showed in Figure 15.

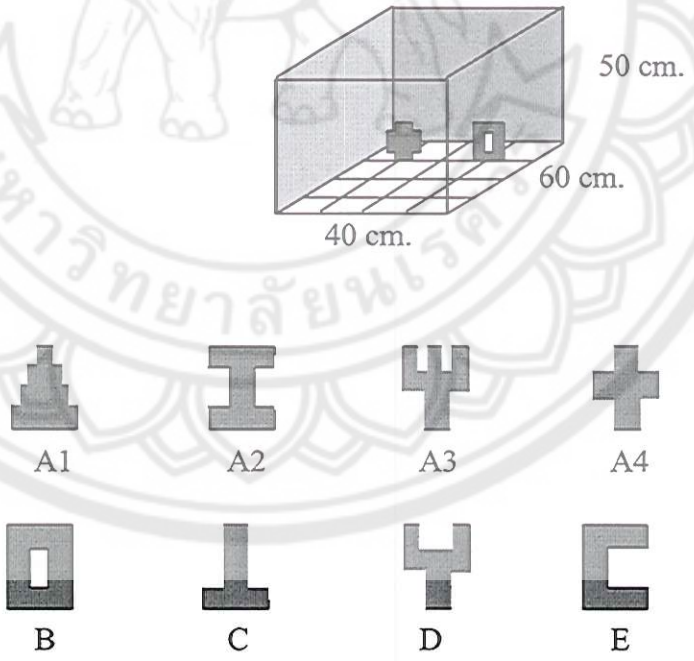

Figure 15 Open field chamber (40x60 cm with walls 50 cm high) (A1 – A4) familiar objects; (B – E) novel objects

Analysis of PCNA, MAP2, MBP and GFAP protein by immunohistochemistry technique

Immunohistochemistry is a technique to detect specific antigen in tissue sections using the principal of antigen-antibody interactions. This method includes unlabeled primary antibody which reacts with specific antigen. The secondary antibody that labeled biotinylated-conjugated was specific binding to primary antibody and then avidin biotinylated horseradish peroxidase complex was added to enhancement the signal. The tissue sections were visualized by adding peroxidase substrate, 3, 3' – diaminobenzidine tetrahydrochloride (DAB).

Antibody

Primary antibody

Mouse monoclonal anti-proliferating cell nuclear antigen; PCNA (MAB424, Millipore, Concord Rd., MA, USA) is expressed during DNA synthesis. PCNA was used as a specific marker to detect cell proliferation. It is identical in all vertebrate such human, mouse, and rat.

Rabbit polyclonal anti-microtubule-associated protein 2; MAP2 (AB5622, Millipore, Concord Rd., MA, USA) was used. MAP2 is one type of microtubule-associated protein which binds with tubulin and maintains microtubule and morphology of the cell. It is expressed during brain development. However, it was found to be expressed in adult brain. It is specific for detection MAP2 of rat, human, and mouse.

Mouse monoclonal anti-glial fibrillary acidic protein; GFAP (MAB360, Millipore, Concord Rd., MA, USA) is a specific marker to investigate the expression of GFAP. GFAP is an intermediate filament protein class III, a main component in astrocytes. It can be used to distinguish between other glial cells and neuronal cell. It is recommended for used with bovine, human, mouse, and rat.

Mouse monoclonal anti-myelin basic protein; MBP (MAB381, Millipore, Concord Rd., MA, USA) was used for a specific marker to determine the expression of myelin basic protein. MBP is a protein that is considerable in process of myelination to produce myelin sheath, envelop around the axon of the neuron. It reacts with MBP from the human, rabbit and rat.

Secondary antibody

Goat anti-mouse biotinylated secondary antibody (BA-9200, Vectastain ABC kit, Vector Laboratories, Burlingame, CA) was used for binding with mouse anti-PCNA, mouse anti-GFAP, and mouse anti-MBP primary antibodies while goat anti-rabbit biotinylated secondary antibody (BA-1000, Vectastain ABC kit, Vector Laboratories, Burlingame, CA) was applied to bind with rabbit anti-MAP2 primary antibody.

Tissue preparation

The rats were scarified by cervical dislocation. Rat brains were removed and fixed in 10% neutral buffered formalin for prevention and maintain the structure and component within the cell. At least 3 days after fixation, rat brains were cut and placed in cassette and immersed in phosphate buffer saline 3 times for 15 min. Tissues were processed using auto tissue processor including, dehydrated through 70% ethanol, 80% ethanol, 85% ethanol, 90% ethanol for 1.30 h, respectively. Followed by, 95% ethanol for 1 h and absolute ethanol 3 times (0.45 min, 30 min and 30 min, respectively). The rat brains were cleared in xylene 2 times (1 h and 0.45 min, respectively) and infiltrated with paraffin wax 2 times for 1.30 h. The tissues were placed in mold and embedded in paraffin wax. Paraffin embedded was cooled and molds were removed. The paraffin blocks were kept at –20°C until sectioning.

Tissue sectioning

The paraffin embedded tissues were sectioned using microtome at the region of hippocampus, area of subgranular and subventricular zones which is guideline from Paxinos and Watson, 1998 into 5 μm thickness. Paraffin-embedded sections were float on warm water (38° C) before mounting on the slides coated with poly–L–lysine hydrobromide (P8920; Sigma, st. Louis, Mo, USA). Sections were allowed to dry at room temperature. Paraffin embedded section were available to use or kept at room temperature.

Immunohistochemistry

The paraffin-embedded sections were deparaffinized with xylene 2 times for 5 min and rehydrated with absolute ethanol 2 times, 95% alcohol 2 times, 80% alcohol and 70% alcohol and distilled water for 5 min each concentration, respectively. Antigens were retrieved by high-temperature heating using microwave at 70p in 0.1 M phosphate buffer saline (PBS, pH 7.4) 3 times for 3 min. Thereafter, sections were allowed to cool down and were washed with PBS 3 times for 5 min. The endogenous peroxidase were blocked with 0.3% hydrogen peroxide (H_2O_2), including 30% H_2O_2, 10% methanol, 1% Triton X-100 diluted in PBS for 30 min. The tissue sections were washed with PBS for 3 times for 5 min. Nonspecific proteins were blocked with 5% normal goat serum diluted in PBS for 2 h at room temperature. The slide sections were incubated with primary antibody comprised of mouse anti-proliferating nuclear antigen; PCNA (diluted 1:100 in PBS containing 1% normal goat serum) for overnight at 4°C, while rabbit anti-microtubule-associated protein 2; MAP2 (diluted 1:750 in PBS containing 1% normal goat serum), mouse anti-glial fibrillary acidic protein; GFAP (diluted 1:1000 in PBS containing 1% normal goat serum), and mouse anti-myelin basic protein; MBP (diluted 1:250 in PBS containing 1% normal goat serum) were incubated for 3 h at room temperature. The slide sections were washed with PBS 3 times for 5 min. Thereafter, incubation with goat anti-mouse biotinylated secondary antibody (PCNA, GFAP and MBP) (diluted 1:200 in PBS containing 1% normal goat serum) while MAP2 were incubated with goat anti-rabbit biotinylated secondary antibody (diluted 1:200 in PBS containing 1% normal goat serum) for 2 h at room temperature. After washing with PBS 3 times for 5 min, the signals were amplified for 30 min with avidin biotinylated horseradish peroxidase complex (ABC) which is prepared before use for 30 min. The slide sections were washed with PBS. Finally, the proteins were visualized with peroxidase substrate, DAB (SK-4100, Vector Laboratories, Burlingame, CA) for 5 min. The immunodetected sections were dehydrated by through; 70% alcohol, 80% alcohol, 2 times at 95% alcohol and 2 times at absolute ethanol for 5 min each concentration and cleared with xylene 2 times for 5 min. Sections were permounted with mounting media and coverslip. Sections were allowed to dry at room temperature and were observed under a light microscope.

Observation and photography

The immunodetected sections were investigated under a light microscope (Nikon Eclipse 80i; Nikon, Bangkok, Thailand, Co., Ltd.) and captured by image capture system (Nikon digital camera DXM 1200C, Nikon, Bangkok, Thailand, Co., Ltd.) that joins with computer. The computer was installed with Nikon ACT-1C for DXM 1200C software (Nikon, Bangkok, Thailand, Co., Ltd.). The images were captured at magnification 20x (GFAP and MBP) while PCNA and MAP2 were made at magnification 40x.

Quantitative analysis for PCNA, MAP2, GFAP immunoreactive cells

Three sections per animal were used in the study. The number of immunoreactive cells was quantified by ImageJ free software supplied by NIH (http://rsb.info.nih.gov/ij/). All analysis, the immunodetected images were adjusted size into 4116x3072 pixels. The cell counting was processed followed by, scale was set up including, adjust known distance into the number is shown in scale bar and unit of length were changed into micron. The value is shown in unit of pixels per micron. Followed by, region of interest (ROI) is set up by configuration random rectangular. The amount of rectangular was based on brain region; in the area of each upper blade and lower blade of dentate gyrus were used three random squares 200x60 μm (GFAP), 80x80 μm (PCNA), and 80x80 μm (MAP2), respectively. In addition, in area of each left and right of subventricular zone were used three random squares 150x150 μm (GFAP), 80x80 μm (PCNA), and 80x80 μm (MAP2), respectively. At phase of cell counting followed by selection plugins, cell counter and chosen load markers. Thereafter, types of cell counter were selected, based on type of cell to be count. The total number of cells is shown behind type of cell.

Semiquantitative analysis for MBP immunoreactivity

The myelin basic protein (MBP) was expressed in myelin sheaths that envelop around the axon of the neurons. This study was investigated in area of cingulate cortex and white matter nearby. The data were represented as optical density. The expression of protein was evaluated using ImageJ free software as same as quantification analysis of immunoreactive cells. MBP immunoreactive images were

adjusted size into 4116x3072 pixels and were changed type into 8-bit. Thereafter, the threshold were analyzed using adjust threshold and image were changed into black and white tone. The optical density is expressed as threshold of difference between expression of specific protein and background.

Statistical analysis

Data are PCNA, MAP2, GFAP immunoreactive cells and MBP immunoreactivity. All data were expressed as mean ± standard error of the mean (S.E.M), considering the control group as 100%. The data from ED-METH binge, ED-METH and AB-METH groups were calculated as the percentage changes from the control group. Statistical analysis between groups was performed using one-way analysis of variance (ANOVA) with post-hoc Dunnett test, a level of *p*-value less than 0.05 were considered statistically significant. Statistical analysis in the same group was performed using independent samples *t*-test, a level of *p*-value less than 0.05 were considered statistically significant. Moreover, the data of behavioral rating scale was expressed as median. Statistical analysis between groups was performed using one-way ANOVA with post-hoc Dunnette test, while ordinal data was performed using Kruskal–Wallis. A level of *p*-value less than 0.05 were considered statistically significant.

Research place

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