CHAPTER III

RESEARCH METHODOLOGY

Instrument and materials

1. Instruments

Autocentrifuger (Biofuge fresco)

Electrophoresis instrument (miniVE, Amersham Biosciences)

Microplate reader (Labsystems iEMS Reader MF)

Microtome (Leica RM 2235)

Novel object recognition test equipment

Tissue embedding machine (Leica EG 1160)

Tissue processing machine (Leica TP 1020)

Video camera (LYD-8080, LYD)

2. Materials

17α-Ethynylestradiol powder (Sigma, St. Louise, MO)

40% formalin (RCI Labscan)

96 well plate (Greiner bio-one)

Acrylamide (Sigma-Aldrich Inc, Louis, USA)

Ammonium Persulfate (APS) (Amresco Inc, Solon, USA)

Anti-BDNF antibody (Santa Cruz Biotecnology Inc, California, USA)

Anti- β-actin antibody (Santa Cruz Biotecnology Inc, California, USA)

Anti-ERα antibody (Abcam Inc, Cambrige, UK)

Anti-ERβ antibody (Abcam Inc, Cambrige, UK)

Anti-rabbit anyibody (Vector Laboratories Inc, Burlingame, USA)

Atropine sulfate

Avidinbiotinylated horseradish peroxidase complexes (ABC) kit (Vector Laboratories Inc, Burlingame, USA)

Bicinchoninic acid protein assay reagent (BCA) kit (Pierce Biotecnology Inc, Rockford, USA.)

Biotinylated anti-rabbit IgG (Vector Laboratories Inc, Burlingame, USA)

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Bovine serum albumin (BSA) blocking buffer (Amresco Inc, Solon, USA)

Cover slips (Menzel-glaser)

ImmPACT™ diaminobenzidine (ImmPACT™DAB) (Vector Laboratories Inc, Burlingame, USA)

Distilled water

Embedding cassette

Eosin (C.V. Laboratories CO., Ltd)

Ethanol (Vidhyasom Co., Ltd.)

Hematoxyline (C.V. Laboratories CO., Ltd)

Heparin

HRP-conjugated chicken anti-goat IgG (Millipore, Billerica, USA)

Liquid nitrogen

Loading dry (Amresco Inc, Cambrige, UK)

Lysis buffer (Invitrogen Corporation, Camarillo, USA)

N, N'-Methylenebisacrylamide (Sigma-Aldrich Inc, Louis, USA)

Paraplast (Leica microsystems)

Pentobarbital (Nembutal®)

Permount (Fisher)

Polyvinylidene difluoride (PVDF) membrane (Pall Corporation, BioTrace)

Propylene glycol (Vidhyasom Co.,Ltd.)

Proteinase inhibitor cocktail (Sigma-Aldrich Inc, Louis, USA)

Protein marker (Gene Direx)

Sodium chloride (MERCK)

Sodium dodecyl sulfate (Sigma-Aldrich Inc, Louis, USA)

TMED (Amresco Inc, Cambrige, UK)

Tris (Amresco Inc, Solon, USA)

Tris-base buffer saline (Amresco Inc, Cambrige, UK)

Tween 20 (Biotect)

Xyline (Zen point)

Plant material and preparation of crude extract

The AR roots were collected from Rayong, Thailand. The voucher specimen of the plant was kept at Faculty of Pharmaceutical Science, Naresuan University, Phitsanulok, Thailand.

The roots of AR were dried by hot air oven at 45 °C for 24 h before milled into coarse powder. After that the dried powdered roots of AR was macerated at room temperature with hexane for 3 days. Then residue was macerated with 95% ethanol for 3 day, and was filtered and extracted again. The crude of AR ethanolic extract was mixed with propylene glycol to stock suspension in a dose of 100 and 1000 mg/kg B.W. The suspension was orally administered to the rats during 7:00 – 8:00 A.M.

Animals

Eight-weeks-old female Wistar rats were purchased from the National Laboratory Animal Center Mahidol University, Nakhon Prathom, Thailand. The rats were acclimatized for at least one week before starting the experiment. They were housed in group of 4-5 animals under a standard light/dark (12:12 h) at constant temperature of 24 ± 1 °C. The animals were allowed free access to food and tab water *ad libitum*. The experiment protocol was approved by the Ethical committee for the Use of Animal, Naresuan University.

Methods

1. Experimental protocols

The rats were examined the estrous cycle by using vaginal cornification assay for three consecutive cycles before treating. All animals were bilaterally ovariectomized or sham-operated under pentobarbital (50 mg/kg B.W.) in aseptic technique during a diestrus phase. Fifteen days after operation, the animals were randomly divided into five groups:

1. Sham group (n = 7), the rats were gavaged with propylene glycol (PG) as a vehicle control.

2. OVX group (n = 7), the OVX rats were gavaged with PG.

3. OVX+AR100 group (n = 7), the OVX rats were gavaged with AR 100 mg/kg B.W.

4. OVX+AR1000 group (n = 7), the OVX rats were gavaged with AR 1000 mg/kg B.W.

5. OVX+17α-ethynylestradiol (EE) group (n = 7), the OVX rats were gavaged with EE 0.1 mg/kg B.W. as a positive control.

All experimental groups were gavaged once daily for 90 days and weighed once a week.

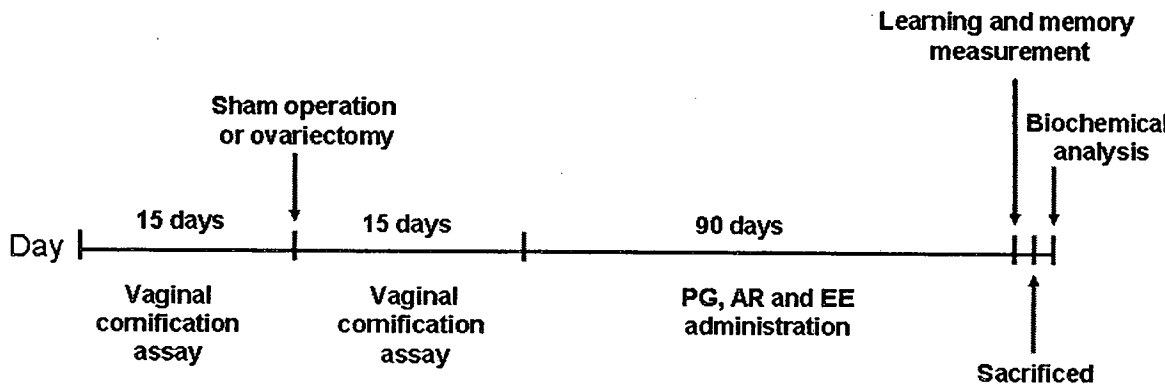


Figure 7 Experimental schedule

2. Preparation of 17α-Ethynylestradiol solution

The 17α-Ethynylestradiol powder was dissolved with small absolute ethanol volume, followed by the addition of deionized water and leave for evaporation of ethanol at room temperature. The solution was kept in the dark bottles at 4 °C (Urasopon, et al., 2007).

3. Vaginal cornification assay

All rats were checked vaginal epithelium daily between 8:00-9:00 A.M. by using disposable pipette which contained 0.9% NaCl solution, and then pushing and injecting 0.9% NaCl solution that was gently into the vagina approximately 2-5 mm depth. After that the fluid in vaginal lumen was sucked up into the pipette and smeared on the microscopic slide to determine the vaginal epithelial cell under a light microscope.

4. Novel object recognition test

The novel object recognition (NOR) test was induced by Ennaceur and Delacuor in 1998. This is a test of dorsal hippocampal function and prefrontal cortex and base on the promise that rodent will explore a novel object more than a familiar one, but only if they remember the familiar one by learning (recognition) and memory process (Ennaceur and Delacour, 1988). This test was performed without external motivation, reward or punishment but a little training or habituation is required (Silvers, et al., 2007). Moreover, it is a widely used model for the investigation of effects on memory and also has been used to test the effects of various pharmacological treatments and brain damage (Goulart, et al., 2010).

4.1 Equipment

The equipment consists of a cubic chamber (100 cm x 100 cm with 50 cm high walls) and three different objects. Object A, B and C was the cylinder, pyramidal and cuboidal shape, respectively. The position of two objects was placed in the centre of the area, 30 cm from each other and 35 cm from the nearest wall of the chamber.

4.2 Acclimatization, training trial and retention test

Before training, each rat was introduced to get acclimatized to the testing environment which is an empty space for 10 min. After acclimatization sessions, the rat was ready for the training session. This session, object A and B were placed in a symmetric position (Figure 8). Each rat was allowed to explore in the box for a total 10 min and considered to be exploring the object when the rat's nose pointed toward the object at a distance $\leq$ 1 cm (Dodart, et al., 2002). The amount of time spent exploring object A and B (T_A and T_B) was recorded manually and calculated as a preference index $[(T_A \times 100)/(T_A + T_B)]$. Following the training period, the rat was removed from the environment for a delay period (10 min). After the delay, the rat was returned to the chamber again, where one of the original object (B) was replaced by new object (C). The recognition index of each rat was calculated from the following formula $(T_C \times 100)/(T_A + T_C)$, where T_A and T_C are the time spent exploring the object A and C, respectively. After each session, the arena and objects were cleaned by 70% ethanol to ensure that behavior of animals was not guided by odor cues.

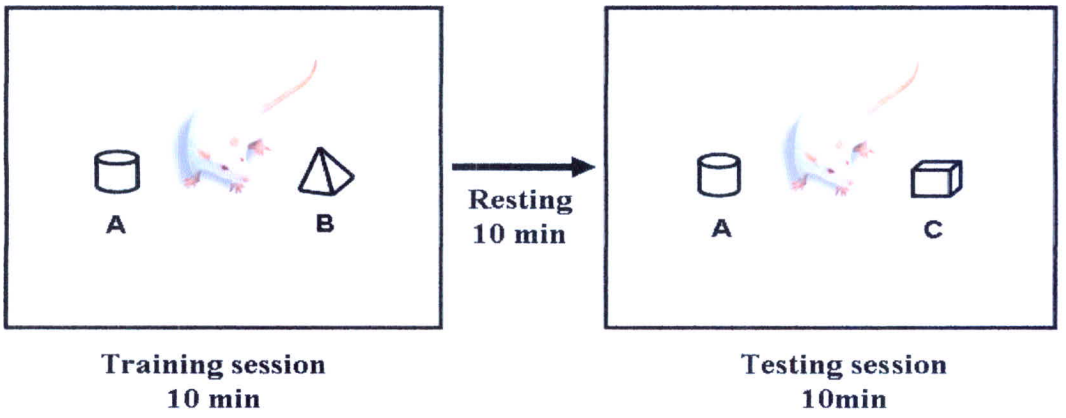


Figure 8 Illustration of the novel object recognition (NOR) test

5. Blood and tissue collection

After study of learning and memory abilities, all experimental animals were sacrificed with pentobarbital (50 mg/kg B.W., intraperitoneal injection). Then, the blood was collected by using a cardiac puncture method and added with heparin to prevent blood clotting. Serum was separated from the blood by centrifugation at 1000 rpm for 10 minutes at room temperature and kept at -80 ^{o}C until analysis. The brain was quickly removed out of the skull after blood collection. The hippocampus and frontal cortex were dissected from left brains and kept at -80 ^{o}C to determine the expression of BDNF and ERs proteins and right brains were fixed in buffered 4% formadehyde to investigate the histological changes.

6. Serum estradiol levels evaluation

Serum estradiol levels was measured using eletrochemiluminescence immunoassay (ECLIA) with Elecsys 2010 automate analyzer (Roche Diagnostics GM6H, Mannhein, Germany) at Faculty of Medicine, Chiang Mai University Hospital, Chiang Mai, Thailand.

An ECLIA was performed according to the procedure described by the manufacturer. Briefly, sample was first incubated with estradiol-specific biotinylated antibody and estradiol-specific antibody labeled with ruthenium to form an immune complex. Next, the streptavidin-coated microparticles were added. The biotin and streptavidin was bound to become a solid phase. After that, sample was drawn to the

surface of the electrode with the help of a magnet and held there temporarily. Unbound substances were removed with Procell. The electrochemiluminescent was generated by voltage application and the resulting light emission was measured by the photomultiplier.

7. Western blot analyses of BDNF and ERs expression

The hippocampus and frontal cortex including rat uterus tissue, a positive tissue for ER subtypes detection, were homogenized in ice-cold lysis buffer with protease inhibitor cocktail. Then the homogenized sample was centrifuged at 10,000 rpm for 10 minutes at 4 °C. After that, the supernatant were transferred to a new tube. The protein content in each sample was measured by using BCA kit.

7.1 Analysis of BDNF expression by western blot technique

Aliquots of sample containing 50 μg of proteins were separated by electrophoresis on 12.5% SDS–PAGE at 100 V for 3 h is followed by electrophoretic transfer to a PVDF membrane at 20 V for 2 h. Next, the blotted membrane were treated with 0.1% tween-20 Tris-buffered saline pH 7.4 (TBS-T) and 5% skim milk for 1 h at room temperature followed by the addition of 1:200 diluted rabbit polyclonal anti-BDNF antibody in TBS-T with 5% skim milk for overnight at 4 °C. The membranes were washed three times for 5 min each in TBS-T and were incubated 30 min at room temperature with 1:200 diluted biotinylated anti-rabbit antibody in TBS-T with 5% skim milk. After washing three times for 5 min each in TBS-T, the blotted membranes were incubated 30 min at room temperature with avidin-biotinylated horseradish peroxidase complexes (ABC kit) to enhancing signal, followed by washing in TBS-T. The immune complexes were visualized using the ImmPACT™ DAB development and followed by rinsed in distilled water for terminate the reaction.

7.2 Analysis of ERs expression by western blot technique

Aliquots of sample containing 70 μg of proteins were separated by electrophoresis on 10% SDS–PAGE at 90 V for 4 h. The separated proteins were eletrophoretically transferred to a PVDF membrane at 20 V for 2 h. The membranes were treated with rapid blocking solution for 30 min at room temperature and followed by the incubation of 1:400 diluted rabbit polyclonal anti- ERα antibody in TBS-T and 1:500 diluted rabbit polyclonal anti-ERβ antibody in TBS-T with 5% skim

milk for overnight at 4°C. Then the membranes were washed three times for 5 min each in TBS-T and were treated with 1:1000 diluted goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody in TBS-T with 5% skim milk for 2 h at room temperature. After washing, the immune complexes were visualized by using the ImmPACT™ DAB development and followed by rinsed with distilled water for terminate the reaction. The illustration of ERs protein band for each sample was shown in the figure 9.

The intensity of each protein band, BDNF and ERs were normalized to their respective loading control, β-actin protein. For the step detection, the blotted membranes were treated with 5% skim milk in TBS-T for 1 h at room temperature. The 1:500 diluted goat polyclonal anti- β-actin antibody in TBS-T with 5% skim milk were added to incubate for overnight at 4°C and followed by washing three times for 5 min each in TBS-T. After that, the blots were incubated with 1:1000 diluted chicken anti-goat HRP-conjugated secondary antibody in TBS-T with 5% skim milk for 2 h at room temperature and washed three times before immune complexes visualizing. The results of protein expression were processed by developing with ImmPACT™ DAB substrate.

The intensity of protein bands were quantified by using Image J software for densitometric analysis and normalized to its respective loading control.

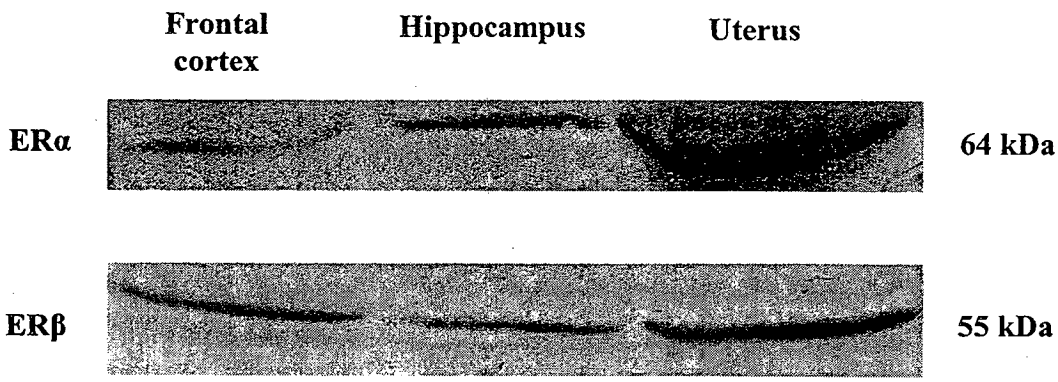
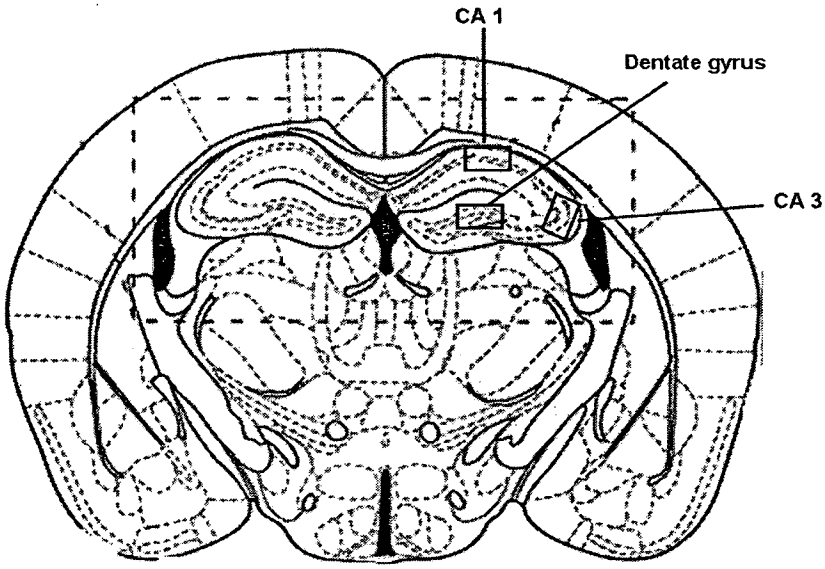


Figure 9 Illustration of ERs protein band of each sample by using western blotting. Uterus tissue was used to indicate actually protein band for ER subtypes expression.

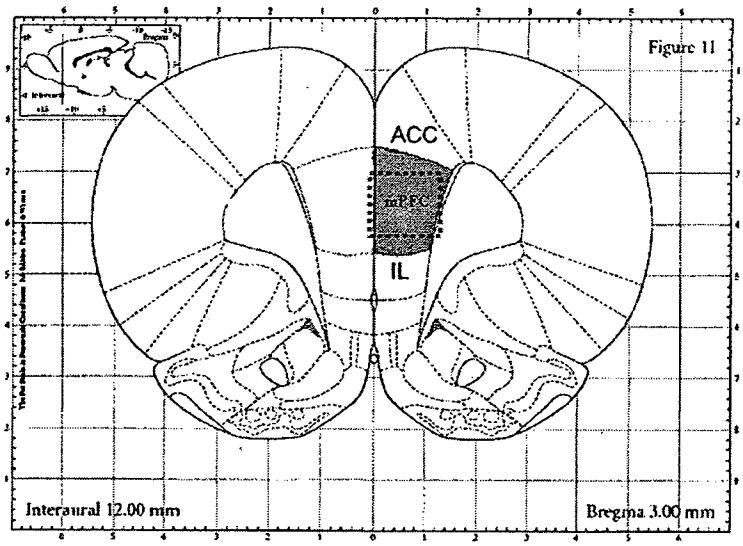
8. Histological analysis

Four brains per group were used to determine the morphological changes. Formalin-fixed brains were dehydrated in ethanol series and embedded in paraffin wax. Each brain block was coronal sectioned at 5 μm thickness and used one out of every 5 sections to determine the density of intact neurons. After allowing tissue slides to dry, the tissue slides were stained with hematoxylin and eosin (H&E) according to standard protocol. The slides were mounted after staining and covered with slide cover slips and analyzed by light microscope (Nikon Eclipse 80i, Nikon) for histological change. All images were photographed and captured with a digital photo camera that attached to the Nikon Eclipse 80i microscope.

The examination of intact neuronal number was performed by using image analysis software (Image-Pro Plus, Media Cybernetics, Silver Spring, USA). The number of intact neurons in the dentate gyrus, CA1 and CA3 sub-regions of hippocampus (approximately -3.14 to 3.30 from bregma) were counted (Figure 10 (A)) in square area 80 x 80 μm^2 under 40x magnifications (Uysal, et al., 2005) and the number of neuron in mPFC (approximately 2.70 to 3.20 from bregma) were counted (Figure 10 (B)) in square area 500 x 500 μm^2 under 20x magnifications (Ni, et al., 1995). The intact neuronal cells were defined as round-shaped, cytoplasmic membrane-intact cells, without any nuclear condensation or distorted aspect (Liu, et al., 2010).



(A)



(B)

Figure 10 Schematic representations of coronal sections illustrating the regions selected for quantification of neuronal viability in the CA1, CA3 and dentate gyrus area of hippocampus (A) and mPFC (B)

Source: Paxinos and Watson, 1998

9. Statistical analysis

The data were expressed as the mean ± standard error of the mean (SEM) and analyzed using a Statistical Package for the Social Sciences (SPSS) software. Comparison among experimental groups was performed by one-way ANOVA with post hoc LSD test. The statistical significance was determined as P-value less than 0.05.