CHAPTER III
MATERIALS AND METHODS

A. Animals

Adult female Wistar rats weighing between 170-190 g (approximately 7 weeks of age) at the beginning of the experiments were obtained from the National Laboratory Animal Center, Mahidol University (NLAC-MU), Thailand. All animals were housed two per cage and were maintained at 25±2°C on 12-h light/dark cycles with lights on at 06:00 a.m. and were given standard rat chow and water *ad libitum*. All procedures were done with the approval of the Animal Use Committee, Faculty of Veterinary Science, Chulalongkorn University.

B. Chemicals

Most of the chemicals used for the high-performance liquid chromatography (HPLC) analysis were purchased from Sigma Chemical Co., St. Louis, MO, USA, except methanol, heptane sulfonate, sodium dihydrogen phosphate, and Na_2·EDTA which were purchased from BDH Chemical Ltd., London, England.

For the Western blot analysis, most of the chemicals were purchased from Bio-Rad laboratories, Hercules, CA, USA, except tris-base and glycine which were purchased from USB Corporation, Cleveland, OH, USA. Glycerol was bought from Cario Erba, Milan, Italy.

Most of the antibodies used in the Western blotting were purchased from Sigma Chemical Co., except the primary antibodies the anti-SERT was purchased from the Calbiochem, EMD Biosciences Inc, Darmstadt, Germany.

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C. Experimental protocols

Experiment 1: To determine the effects of estrogen on the anxiety-like behaviors of the female rats in the ETM (Figure 6)

Female Wistar rats were divided into 3 groups of 11 animals each:

1. Ovariectomized with vehicle treated group (Ovx)
2. Ovariectomized with estrogen supplementation group (Ovx+E_2)
3. Sham in proestrous phase group (Pro)

The rats were tested with ETM and open field at four weeks after ovariectomy.

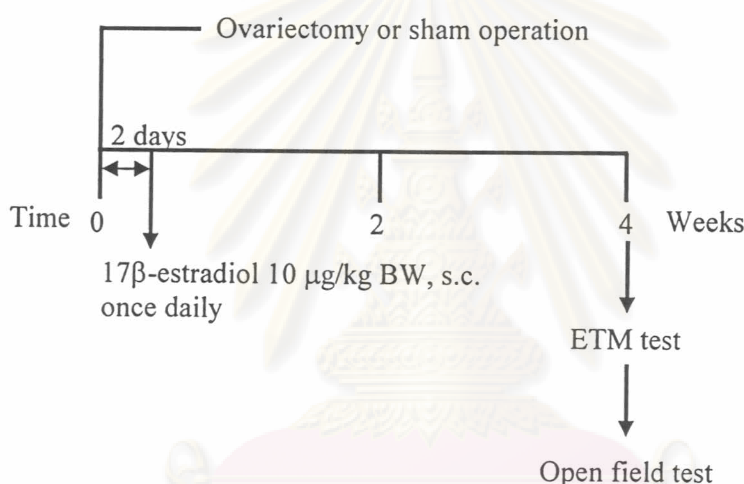

Figure 6. Diagram of the experimental protocol 1, BW = body weight, ETM = elevated T-maze, s.c. = subcutaneous injection

Experiment 2: To examine the effects of estrogen on the serotonergic neurotransmission after the ETM test

After behavioral tests, the rats were sacrificed with halothane and their brains were removed for measurement of 5-HT and 5-HIAA levels in frontal cortex, nucleus accumbens, septum, amygdala and hippocampus by HPLC-EC technique using 8 brains in each group. For the remaining brains used for measurement of SERT protein levels in frontal cortex, nucleus accumbens, septum, amygdala and hippocampus and TPH protein levels in midbrain with Western blot analysis.

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Experiment 3: To examine the effects of estrogen on the postsynaptic serotonin receptor function in the anxiety-like behaviors tested with the ETM

Rats were divided into 6 groups of 6-11 animals each:

1. Ovariectomized with vehicle treated group (Ovx+Veh)
2. Ovariectomized with ritanserin (5-$HT_{2A/2C}$ receptor antagonist) treated group (Ovx+Rit)
3. Ovariectomized with estrogen supplementation and vehicle treated group (Ovx+E_2+Veh)
4. Ovariectomized with estrogen supplementation and ritanserin (5-$HT_{2A/2C}$ receptor antagonist) treated group (Ovx+E_2+Rit)
5. Sham in proestrous phase with vehicle treated group (Pro+Veh)
6. Sham in proestrus phase with ritanserin (5-$HT_{2A/2C}$ receptor antagonist) treated group (Pro+Rit)

The protocol is similar to the first experiment, only that the vehicle (10% DMSO) or ritanserin (5-$HT_{2A/2C}$ receptor antagonist) at a dosage of 0.3, 1, or 3 mg/kg were injected intraperitoneally 30 min before testing with ETM and open field (Figure 7).

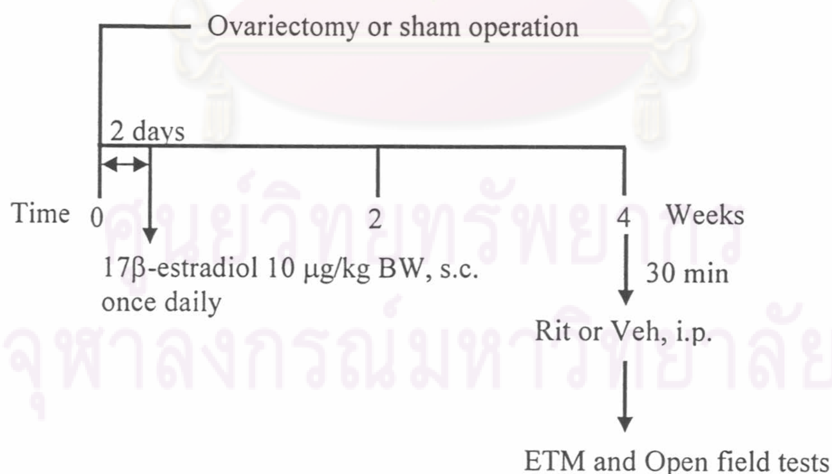

Figure 7. Diagram of the experimental protocol 3, BW = body weight, ETM = elevated T-maze, i.p. = intraperitoneal injection, Rit = ritanserin, s.c. = subcutaneous injection, Veh = vehicle

D. Methods

1. Animal preparation

After 7-day adaptation period, the rats were randomly assigned into 3 groups: Ovx, Ovx+E_2, or Pro groups. For Ovx and Ovx+E_2 groups, the rats were anesthetized with halothane and both ovaries were removed. For Pro group, the same operation procedure was performed except that the ovaries were kept intact. In these Pro rats, vaginal smears were taken daily for 2 weeks also on the day of behavioral testing for examination of consistency and determination of different stages of estrous cycle. Rats at proestrous phase and showing three consecutive 4-day cycles were used on the behavioral test day.

For Ovx with estrogen supplementation, replacement regimens were started 2 days after ovariectomy by injecting 17β-estradiol (10 μg/kg in propylene glycol) subcutaneously into the dorsal region of the neck, once daily. In Pro and Ovx groups, rats were injected by an equivalent volume of the vehicle. The dose of estrogen was considered a physiological dose because it induced lordosis behaviors similar to that observed at estrus (Diaz-Veliz *et al.*, 1991), and daily treatments were used to maintain levels of plasma estrogen.

2. Measurement of body weight, food intake and uterine weight

Body weights (BW) and food intake of the animal were determined daily. Uterine weight (UW), the indicator of ovarian hormones deficiency after removal of ovaries, was determined immediately after sacrifice.

The daily weight gain (DWG), the daily food intake (DFI), and the percentage of uterine-to-body weight ratio (% UW/BW) were calculated, as follows:

DWG = final BW - initial BW/28

DFI = summation of food intake/28

% UW/BW = (UW(g)/BW(g)) × 100

3. Vaginal smears

Estrous cycle phases were determined for 2 weeks before ETM test by vaginal smear according to the method of Marcondes *et al.* (2002). Every morning between 10:00 and 11:00 a.m., one drop of vaginal secretion was collected with a

plastic pipette filled with 10 μl of normal saline (0.9% NaCl) by inserting a clean tip into the rat vagina, but not deeply. Vaginal fluid was dropped and smeared on glass slides. Unstained material was observed under a light microscope with 10x and 40x objective lenses. Three types of cells could be recognized: round and nucleated ones are epithelial cells; irregular ones without nucleus are the cornified cells; and the little round ones are the leukocytes. The proportion among them was used for the determination of the estrous cycle phases (Long and Evans, 1922; Mandl, 1951). A proestrous smear consists of a predominance of nucleated epithelial cells; an estrous smear primarily consists of anucleated cornified cells; a metestrous smear consists of the same proportion among leukocytes, cornified, and nucleated epithelial cells, and a diestrous smear primarily consists of a predominance of leukocytes (Long and Evans, 1922; Mandl, 1951).

4. Behavioral assessment

4.1 Elevated T-maze test

4.1.2 Apparatus

The ETM (Figure 8), validated model for measurement of anxiety (Graeff *et al.*, 1993; Mora *et al.*, 1997; Viana *et al.*, 1994; Zangrossi *et al.*, 1999), is made of wood and consisted of two open arms (50 cm × 10 cm) and one enclosed arm (50 cm × 10 cm × 40 cm) perpendicular to the open arms; the arms were connected by a square (10 cm × 10 cm) and the apparatus was elevated 50 cm above the floor. To avoid falling from the open arms, they were surrounded by a 1 cm high Plexiglas rim.

4.1.3 Procedure

The experiments were conducted between 09:00 and 12:00 a.m. The test session consisted of three inhibitory avoidance trials and one escape trial held at 30-s intervals according to the method of Graeff *et al.* (1993). Between the trials, the animals were placed in the Plexiglas cage. On the first three inhibitory avoidance trials, each animal was placed at the distal end of the enclosed arm facing the center of the maze. The baseline latency was defined as the time(s) required for the rat to exit the enclosed arm (defined as all the four paws outside the arm). The same

measurement was repeated in two subsequent trials (avoidance 1 and 2). Following avoidance training, the escape trial was done. The animal was placed at the end of the right open arm facing the center of the maze and the time the animal took to exit this arm with the four paws was recorded (escape time). Between each rat, the maze was carefully wiped with 20% alcohol.

The inhibitory avoidance task represented conditioned fear, while one-way escape from the open arm represented unconditioned fear.

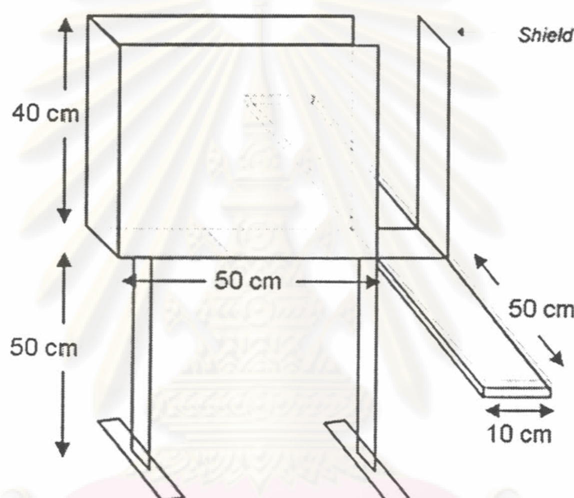

Figure 8. The ETM consists of three arms of equal dimension (50 × 10 cm) elevated 50 cm from the floor. One of these arms was enclosed by lateral walls (40 cm high) and was positioned perpendicularly to the two opposed open arms.

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4.2 Open field test

4.2.1 Apparatus

The open field (Figure 9A), for measuring locomotor activity, is a wooden box (76 cm long × 57 cm wide × 35 cm high) with a 48-square grid floor (6 × 8 squares, 9.5 cm per side). All but the 24 peripheral squares are considered as inner zone (Figure 9B).

4.2.2 Procedure

After the ETM session, the animals were tested in the open field for 5 min to measure locomotor activity. The open field test was used in accordance with the methods described by McCarthy *et al.* (1995). The numbers of total crosses that the rat made during the 5 min in this task were recorded as the locomotor activity. Times spent in the inner zone were considered indicative of anti-anxiety behavior in the rodent. The experiments were recorded by a video camera for later analyzed.

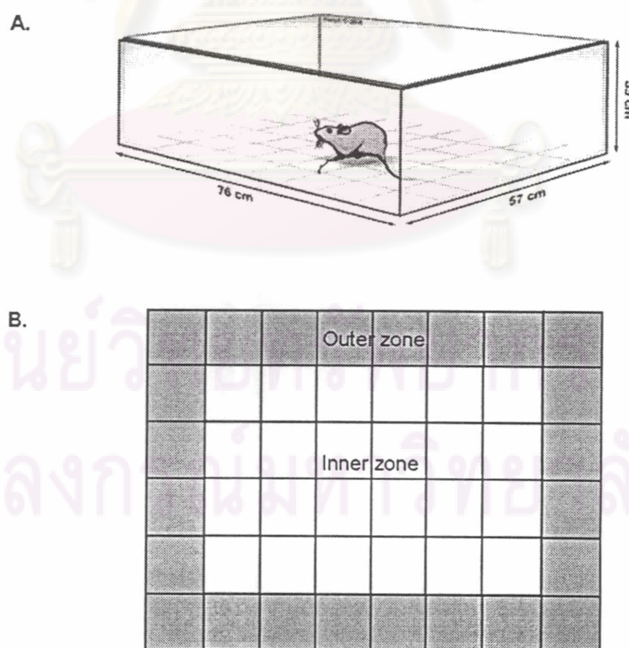

Figure 9. (A) The open field apparatus was a wooden box (76 cm long × 57 cm wide × 35 cm high) with a 48-square grid floor (6 × 8 squares, 9.5 cm per side). (B) Floor of the maze was divided into 2 zones, 24 peripheral squares considered as outer zone (gray area) and remaining 24 inner squares as inner zone (white area).

5. Brain dissection

After the open field test, the rats were euthanized and their brains were rapidly removed, frozen in liquid nitrogen, and stored at -70°C. Brains were isolated into each area (followed the instruction of Heffner *et al.*, 1980) and placed in 1.5 ml Eppendorf tubes. Briefly, a frozen brain was placed on its dorsal surface in the trough of the brain cutting block (Figure 10). All of the following procedure was done on ice. Razor blades were carefully inserted through the cutting channels slicing the brain at right angles to the sagittal axis. This initial razor blade slices through the coronal plane of the brain at the level of the anterior commissure. The position of the initial razor blade served as a reference point from which brain sections were obtained. Total of eight razor blades were inserted anterior or posterior to the first blade as shown in Figure 10. The brain was thus divided into 8 sections (Figure 11). The razor blades were removed from the block with coronal brain slices adhering to their surfaces and were placed on a glass plate suspended on ice. Brain regions were then bilaterally dissected from these slices. The frontal cortex, nucleus accumbens, septum, amygdala, and hippocampus were used for HPLC and Western blot analysis.

The frontal cortex was consisted of the frontal poles, cortical tissue from section 1 (see Figure 11.1), as well as the cortical tissue superior to the rhinal sulcus from sections 2 and 3 (Figures 11.2 and 11.3). The nucleus accumbens was dissected from the rostral surface of the third brain section (Figure 11.3). The septum was dissected from the caudal surface of the fourth brain section (Figure 11.4) based on their distinct morphological appearances. The amygdala included the tissue lateral to both portions of the hypothalamus (sections 5 and 6) and ventral to the rhinal sulcus. The hippocampus was separated from the midbrain and overlying cerebral cortex from sections 7 and 8 (Figures 11.7 and 11.8) based on its distinct morphological appearance. For the midbrain, the two most caudally placed razor blades were not inserted resulting in a total of seven brain sections. After the cerebellum is removed from the brainstem, the midbrain and pons-medulla are separated by a cut at the level of the inferior colliculi.

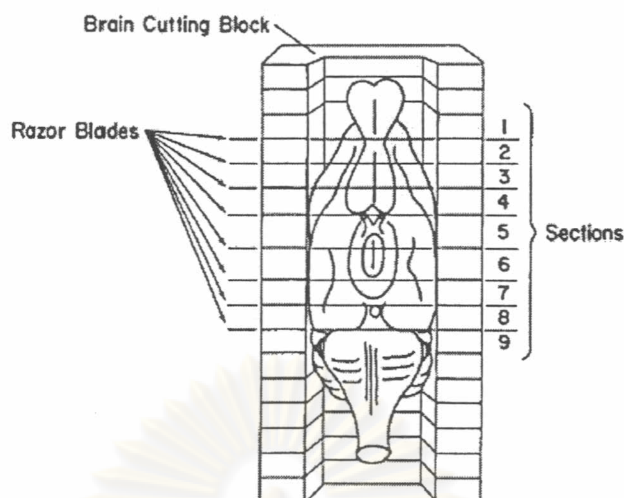

Figure 10. Diagrammatic representation of brain cutting block illustrating orientation of brain and placement of razor blades to obtain coronal brain sections. The numbers on the right refer to brain sections (Heffner *et al.*, 1980).

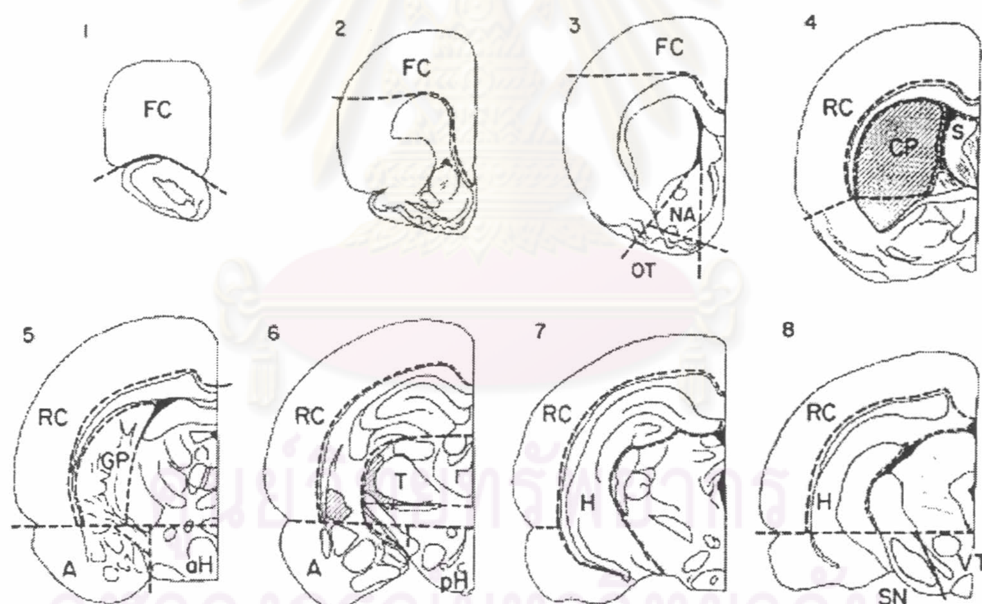

Figure 11. Diagrammatic representation of coronal brain sections from which brain regions are dissected. Dotted lines indicate borders of brain regions. FC, frontal cortex; NA, nucleus accumbens; OT, olfactory tubercle; S, septum; CP, caudate putamen; RC, remaining cortex; GP, globus pallidus; aH, anterior hypothalamus; pH, posterior hypothalamus; A, amygdala; T, thalamus; SN, substantia nigra; VT, ventral tegmentum; H, hippocampus. Numbers correspond to brain sections shown in Figure 10 (Heffner *et al.*, 1980).

6. Neurochemical analysis

The isolated brains were sonicated in the cold 0.1 M perchloric acid containing 3,4-dihydroxy-benzyl-amine hydrobromide (DHBA), as an internal standard. Before the centrifugation, 20 µl of brain homogenate was collected for protein determination. After that samples were centrifuged at 10,000x g (Biofuge 22R, Heraeus Instruments, Germany) for 30 min at 4°C. The supernatants were collected and stored at -20°C for further analysis of monoamine neurotransmitters using HPLC with electrochemical detector (HPLC-EC).

6.1 HPLC-analysis

HPLC-EC, a glassy carbon working electrode and amperometric control (Bioanalytical systems, West Lafayette, IN, U.S.A.) were used to quantify neurotransmitter levels. A Shimadzu Model LC-10 AD pump (Kyoto, Japan) was connected to a Rheodyne (Cotati, CA, U.S.A.) injector, equipped with a 20 µl fixed loop and a 15-cm phenomenex® column (Phenomenex, USA), packed with 5-µm particles. The mobile phase solution was composed of 1 mM Heptane sulfonate, 100 mM Sodium dihydrogen phosphate, 1 mM Na_2·EDTA and 5% Methanol, adjusted to pH 4.1 with saturated citric acid. The mobile phase was filtered through a 0.22-µm filter, degassed by ultrasonic agitation and pumped at a flow-rate of 1.0 ml/min. The amperometer was set at a positive potential of 0.700 V with respect to the Ag/AgCl reference electrode, with a sensitivity of 2 nA. The supernatant (40 µl) from the brain was injected into the HPLC-EC system to detect the 5-HT and its metabolite 5-HIAA.

Delta 5.0 software (Digital Solutions, Margate, QLD, Australia) was used to analyze the chromatography data.

6.2 Analytical procedures

Standard solutions at different concentrations were injected into HPLC system. The retention time was evaluated by injecting the standard serotonin and its metabolite individually and by the injection of a standard mixture (Figure 12). The concentrations of transmitters and metabolites were calculated by reference to standards and internal standard using peak integration and expressed as pmol/mg

protein of brain tissue. Protein concentrations of various brain regions were determined by the method of Lowry *et al.* (1951).

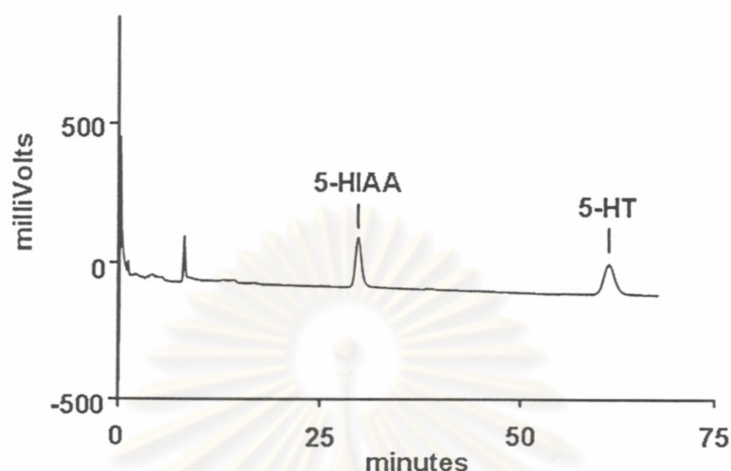

Figure 12. The chromatogram represents peaks of standard 5-HT and 5-HIAA measured by HPLC-EC. The retention times of 5-HIAA and 5-HT were approximately 29.6 and 59.5 minutes, respectively.

7. Western blot analysis

7.1 Electrophoresis

The frontal cortex, nucleus accumbens, septum, amygdala, and hippocampus were dissected similar to previously described method modified from Heffner *et al.* (1980). All steps were performed on ice. Each 1 g of dissected brain was immediately homogenized in 200 μl of 50 mM Tris (pH 7.5) and 20 mM 2-β-mercaptoethanol and centrifuged at 12000x g (Biofuge 22R, Heraeus Instruments, Germany) for 10 min. The supernatant was removed and stored at -70°C for TPH protein analysis. The pellet was stored at -70°C until solubilization and assay for SERT protein with Western blot analysis. Pellets containing membrane-bound proteins were obtained and resuspended in 10 mM Tris and 1 mM EDTA, pH 7.2, containing protease inhibitor cocktail and further homogenized with a handheld pestle and mortar. Sample protein concentrations were determined by the Lowry method (Lowry *et al.*, 1951). Samples (50-75 μg protein/lane) were resolved by 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis using vertical minigel system (Bio-Rad). Each sample was separated in triplicate.

7.2 Immunodetection for TPH and SERT

Subsequent to separation, proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Hybond-P; Amersham Biosciences, Arlington Heights, IL) in Tris-glycine transfer buffer. Blotted membranes were then blocked with 5% nonfat powdered milk in Tris-buffered saline for 4 hour at room temperature. For identification of proteins, membranes were washed (2 × 5 min) and incubated overnight at 4°C with the primary antibodies diluted in 1% milk. The primary antibodies were monoclonal anti-rabbit TPH (clone WH-3) and rabbit anti-rat SERT at the dilution of 1:300 and 1:3000, respectively. Following the primary antibody incubation, the membranes were washed and then incubated for 2 h in 1:1000 horseradish peroxidase-conjugated secondary antibody at room temperature. This incubation step was terminated with several washes and the immunoreactive protein bands were visualized using enhanced chemiluminescence (ECL Plus; Amersham Biosciences) according to manufacturer's instructions. Membranes were exposed to film (Hyperfilm-ECL; Amersham Biosciences) for times adequate to visualize chemiluminescent bands. Blots were reprobed with 1:50,000 monoclonal anti-β-actin (clone AC-15) and normalized to verify equivalent protein loading. Comparisons were made with known molecular weight standards. Differences in protein immunoreactivity between treatments were determined by scanning densitometry in proportion to β-actin immunoreactive bands (Scion Image; Scion Corporation, Frederick, MD).

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8. Measurement of protein concentration

Total protein concentrations used in both HPLC and Western blot analyses were measured according to the Lowry's method (Lowry *et al*., 1951).

8.1 Reagents

Reagent A was 2% Na_2CO_3 in 0.1 M NaOH. Reagent B was 4% sodium tartrate. Reagent C was 2% $CuSO_4 \cdot 5H_2O$. Reagent D was freshly prepared from mixing of reagent A, B, and C at a ratio of 200:1:1. Folin-cioculteau phenol reagent was diluted 1:2 in H_2O before use. Bovine serum albumin (BSA) was used as a standard.

8.2 Procedure

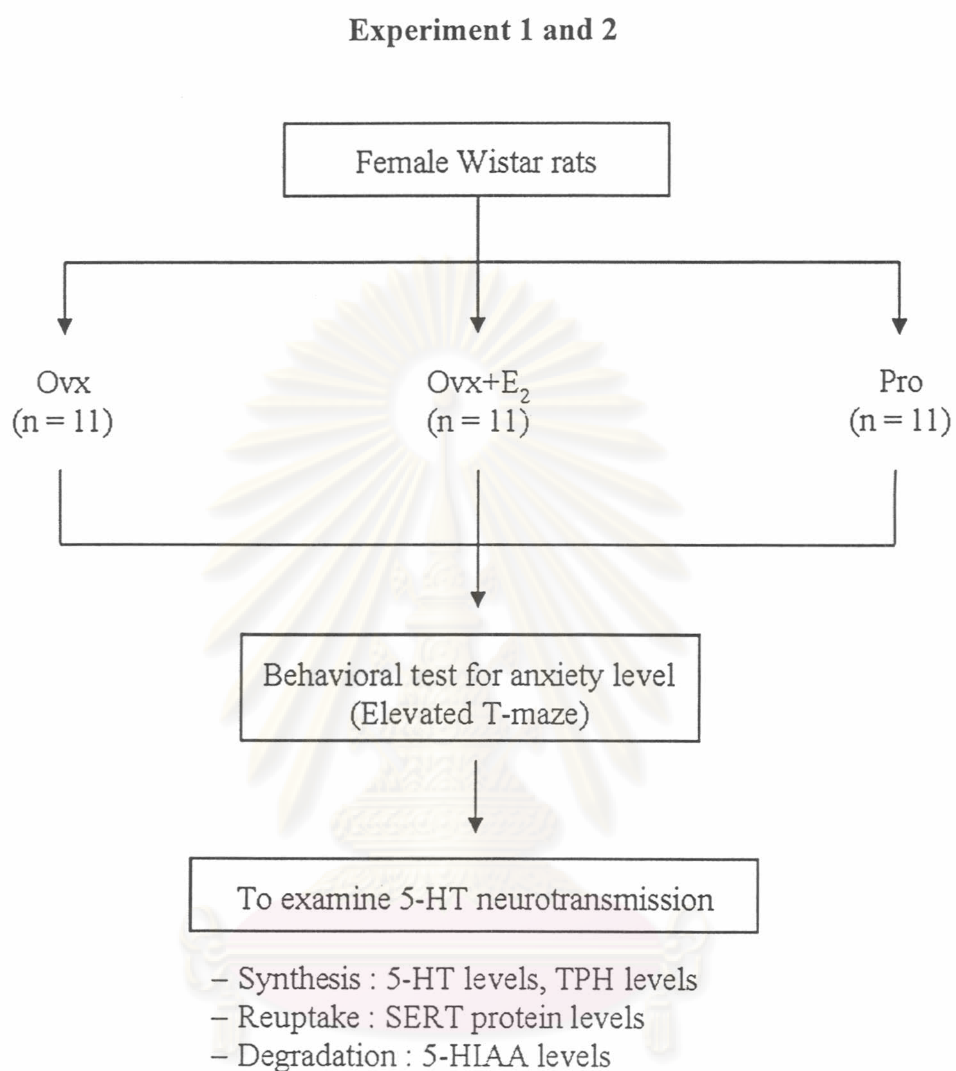
Sample or standard 100 μl was added with 100 μl of reagent A and followed by 1 ml freshly Reagent D. Diluted Folin reagent 100 μl was added, rapidly mixed, and allowed to interact for 30 min at room temperature. The absorbance was then measured at 750 nm. A standard curve of absorbance was plotted as a linear correlation for determination of the unknown protein concentration.

9. Statistical analysis

The avoidance latency in the ETM was analyzed by two way analysis of variance (ANOVA) with treatment as the independent factor and trials (baseline, avoidance 1 and 2) as the dependent factor. In case of significant effect of treatment or of treatment versus trials interaction, data were analyzed by one-way ANOVA followed by the Duncan post hoc test. Other data were submitted to one-way ANOVA followed by the Duncan post hoc test. The linear regression analyses were carried out between rat anxiety parameters and the levels of serotonin and its metabolite levels in all measured brain regions. In all cases, a value of $P < 0.05$ was considered significant.

E. Summary of experimental design

Experiment 1 and 2



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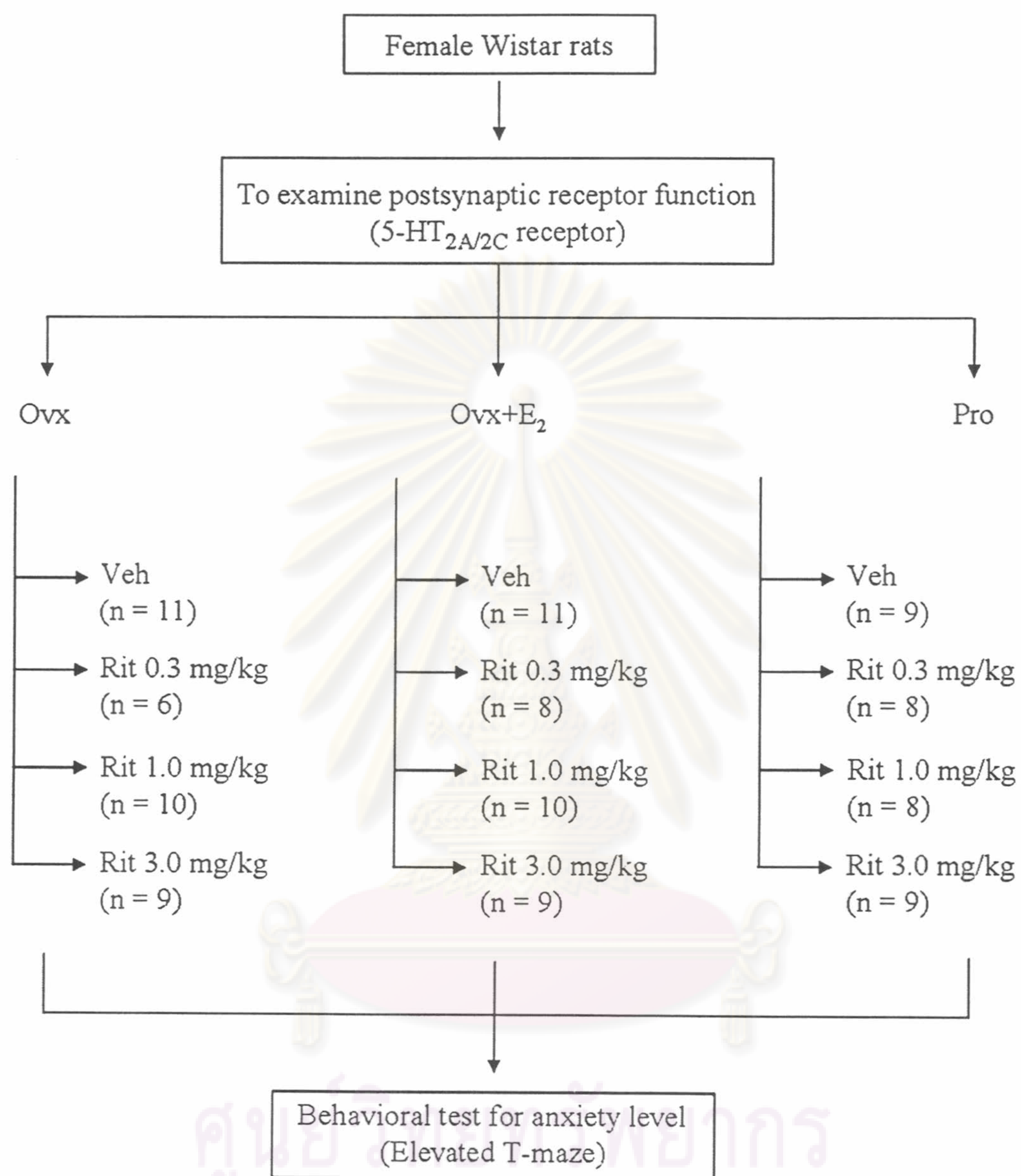
Experiment 3



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