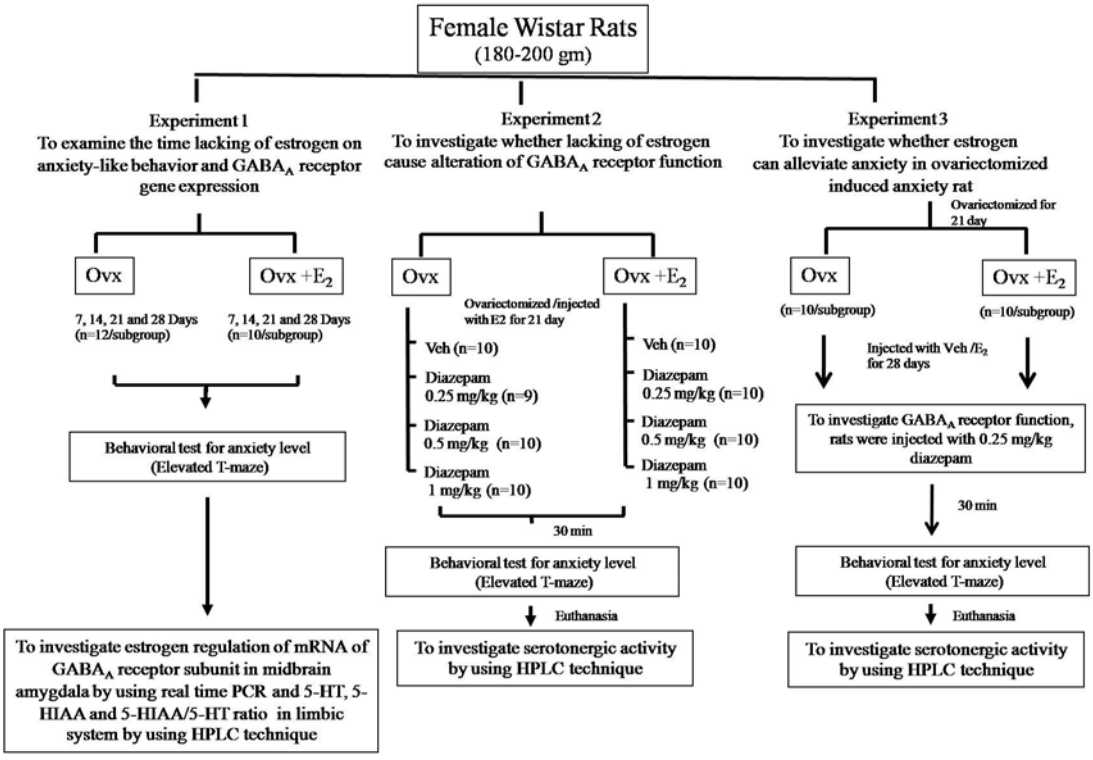
CHAPTER III
MATERIALS AND METHODS

In order to test the hypotheses, this study was divided into 3 experiments as follows:

Experiment 1: To investigate the effects of estrogen deprivation on anxiety-like behaviors, $GABA_A$ receptor subunits gene expression and serotonergic activity in brain associated with anxiety in ovariectomized rats

Experiment 2: To investigate whether lacking of estrogen causes alterations of $GABA_A$ receptor function and whether these alterations affect serotonergic activity in brain associated with anxiety

Experiment 3: To investigate whether estrogen can alleviate anxiety-like behavior in ovariectomized rat and whether the $GABA_A$ receptor function is modulated by estrogen, which in turn modulates the serotonergic activity in brain areas related to anxiety



Summary of experimental design

A. Animals

Female Wistar rats weighing 180-200 gm at the beginning of the experiments were obtained from National Laboratory Animal Center, Mahidol University (NLAC-MU), Thailand. All animals were housed in pair in shoebox cage (23 cm wide x 40 cm long x 20 cm high) and maintained at 12h light/dark cycle (lights on at 0600 h; light intensity approximately 150 lx) at room temperature ($25\pm2^{o}C$). Standard rat chow and water were supplied *ad libitum* (CP082, Perfect Companion Group Co. Ltd., Thailand). All procedures were done according to the National Institutes of Health Guide for care and used of Laboratory animals under the approval of Animal Used Committee, Faculty of Veterinary Science, Chulalongkorn University (Protocol number 0831056 and 1031030).

B. Chemicals

The chemicals for high-performance liquid chromatography (HPLC) analysis used in this study were purchased from Sigma Chemical Co., St. Louis, U.S.A. For $GABA_A$ gene expression analysis, the Aurum™ total RNA fatty and fibrous kit and the iScript™ select cDNA synthesis kit were purchased from BioRad Laboratory, Hercules, U.S.A and the power SYBR® Green master mix used for Real-Time-Polymerase Chain Reaction (PCR) analysis was purchased from Applied Biosystems, Foster City, California, U.S.A. In some behavioral tests utilizing the benzodiazepine agonist, diazepam was purchased from Atlantic Laboratories Corp., Co., Ltd., Bangkok, Thailand.

C. Experimental protocols

Experiment 1: To investigate the effects of estrogen deprivation on anxiety-like behaviors, $GABA_A$ receptor subunits gene expression and serotonergic activity in brain associated with anxiety in ovariectomized rats

After 7-day adaptation period, all rats were bilaterally ovariectomized under anesthesia (Isoflurane; Minrad Inc., Bethlehem, Pennsylvania, U.S.A.), and were

randomly assigned into 2 groups: ovariectomized-rat (Ovx) and ovariectomized-rat with estrogen replacement (E_2). For E_2 groups, replacement regimen was started 1 day after ovariectomy by injecting 17β estradiol (1 μg/kg in propylene glycol) subcutaneously into the dorsal region of the neck, once daily. This dose was selected based on previous study as the smallest dose that contained anxiolytic effect in ovariectomized rats (Kalandakanond-Thongsong et al., 2012). In Ovx groups, rats were injected with an equivalent volume of the propylene glycol. At 7, 14, 21 or 28 days after ovariectomy, the rats from both groups were randomly selected for behavioral test. Accordingly, the E_2 groups were designated as E7, E14, E21 and E28; while, the Ovx groups were designated as Ovx7, Ovx14, Ovx21 and Ovx28, respectively.

After behavioral test, the rats from each group were sacrificed with an overdosage of isoflurane. Their brains were rapidly removed, frozen in liquid nitrogen and store at -80ºC for further analysis with HPLC-EC or real time PCR. Moreover, the uteri were weighed and used as an indicator of estrogen deficiency after ovariectomy.

Experiment 2: To investigate whether lacking of estrogen causes alteration of $GABA_A$ receptor function and whether these alterations affect serotonergic activity in brain associated with anxiety

In this experiment, all procedures were done similar to those of experiment 1. According to the experiment 1, anxiety can be uniformly induced by 21-day ovariectomy; this time point was then used for further experiment. In this experiment, the female Wistar rats were divided into ovariectomized (Ovx) and ovariectomized with estrogen administration (E_2) groups. At 21 days after ovariectomy, the rats from each group were further divided into 4 subgroups receiving diazepam at the dosages of 0, 0.25, 0.5 or 1 mg/kg. The vehicle (0.9 % normal saline) or various doses of diazepam were injected subcutaneously 30 min before testing with elevated T-maze. After behavioral test, the rats were sacrificed with an overdosage of isoflurane. Their brains were rapidly removed, frozen in liquid nitrogen and store at -80ºC for further analysis with HPLC-EC for the measurement of 5-HT and its metabolites, 5-HIAA.

Experiment 3: To investigate whether estrogen can alleviate anxiety-like behavior in ovariectomized rat and whether the $GABA_A$ receptor function is modulated by estrogen, which in turn modulates the serotonergic activity in brain areas related to anxiety

In this experiment, all rats were bilaterally ovariectomized under anesthesia (isoflurane) using the same procedure as previously described. After ovariectomy for 21 days, the rats were randomly assigned into 2 groups: ovariectomized-rat (Ovx) and ovariectomized-rat with estrogen replacement (E_2). For E_2 groups, the rats were injected with 17β estradiol (1 μg/kg 17β-estradiol in propylene glycol) subcutaneously into the dorsal region of the neck, once daily for 28 days. This period was selected as it can prevent anxiety in ovariectomized rats (Pandaranandaka et al., 2006; 2009; Kalandakanond-Thongsong et al., 2012). On the behavioral test day, the rats in each group were further divided into 2 subgroups receiving vehicle or diazepam (0.25 mg/kg) 30 min before testing with elevated T-maze. The dose of diazepam was selected based on the results from experiment 2.

After behavioral test, the rats were sacrificed with an overdosage of isoflurane. Their brains were rapidly removed, frozen in liquid nitrogen and store at -80ºC for further analysis with HPLC-EC for the measurement of 5-HT and its metabolites, 5-HIAA.

D. Methods

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

Body weights (BW) and food intake of the rats were measured daily to determine the physiological changes. The daily weight gain (DWG), the percent change of body weight (%change of BW) and the daily food intake (DFI) were calculated using following equations;

DWG (gm/d) = [final BW (gm) – initial BW (gm)] / day of experiment (days)

% change of BW = [[final BW (gm) - initial BW (gm)] / initial BW (gm)] x 100

DFI (gm/rat/d) = [Total food intake (gm) /2] / day of experiment (days)

The uterine weight (UW) was normalized to the body weight and presented as the percentage of the uterine weight to body weight (%UW/BW) using following equation;

%UW/BW = [UW (gm) / BW (gm)] x 100

2. Behavioral assessment

In order to eliminate the effect of previous exposed to the behavioral tests, each rat was submitted to behavioral test including ETM and open field only once. The behavioral tests were conducted between 0900 am and 1200 pm in a dimly illuminated room with the light intensity of approximately 15-20 lx in the apparatuses.

2.1 Elevated T-maze test

The ETM, a validated model for measurement of anxiety (Graeff et al., 1993; Mora et al., 1996; Zangrossi and Graeff, 1997; Zangrossi et al., 1999), was made of black wood, consisted of two open arms (50 cm × 10 cm) and one closed arm (50 cm × 10 cm × 40 cm) and elevated 50 cm above the floor (Figure 3-1). To prevent rats from falling, the open arms were surrounded by a 1 cm high Plexiglass rim. The test session consisted of three inhibitory avoidance trials and three escapes trials held at 30-s intervals according to the method of Graeff et al. (1993). Between the trials, the animals were placed in the Plexiglass cage. In the inhibitory avoidance trials, each animal was placed at the distal end of the closed arm facing the center of the maze. The baseline latency was defined as the time(s) required for the rat to leave the closed arm (defined as all the four paws outside the arm). The same measurement was repeated in two subsequent trials (designated as avoidance 1 and 2). Following the inhibitory avoidance trials, the escape trials were done. The animal was placed at the end of the right open arm facing the center of the maze and the time the animal enter to the closed arm with the four paws was recorded. For all tasks, a cutoff time of 300 s was established. Between each rat, the maze was carefully wiped with a wet towel.

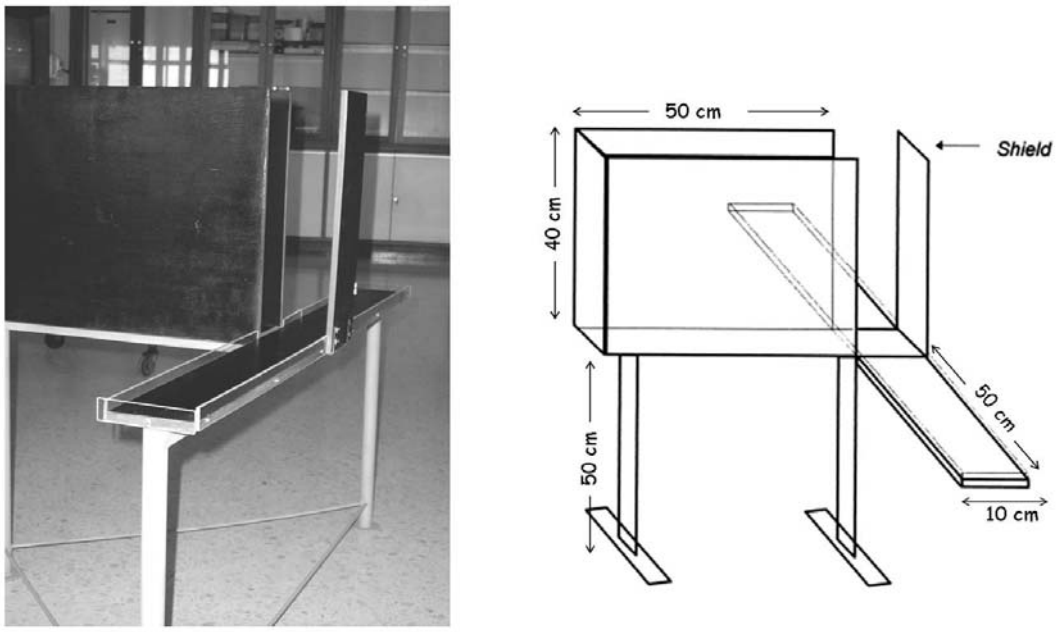


Figure 3-1 The elevated T-maze composed of two open- and one closed-arms of equal dimension (10 x 50 cm), connected by the center platform (10 x 10 cm). The closed-arm was enclosed by a 50-cm wall, and the maze was elevated 50 cm above the floor.

2.2 Open field test

After the ETM session, the animals were tested in the open field arena for 5 min to measure locomotor activity. The open field test was done in accordance with the methods described by McCarthy et al. (1995). The open field was a black wooden box (76 cm long × 57 cm wide × 50 cm high) with a 48-square grid floor (6 × 8 squares, 9.5 cm per side) (Figure 3-2). The numbers of total line crossed that the rat made during the 5 min in this task were counted and interpreted as the locomotor activity. The experiments were recorded by a digital camcorder for later analysis. Between each rat, the apparatus was completely cleaned with a wet towel.

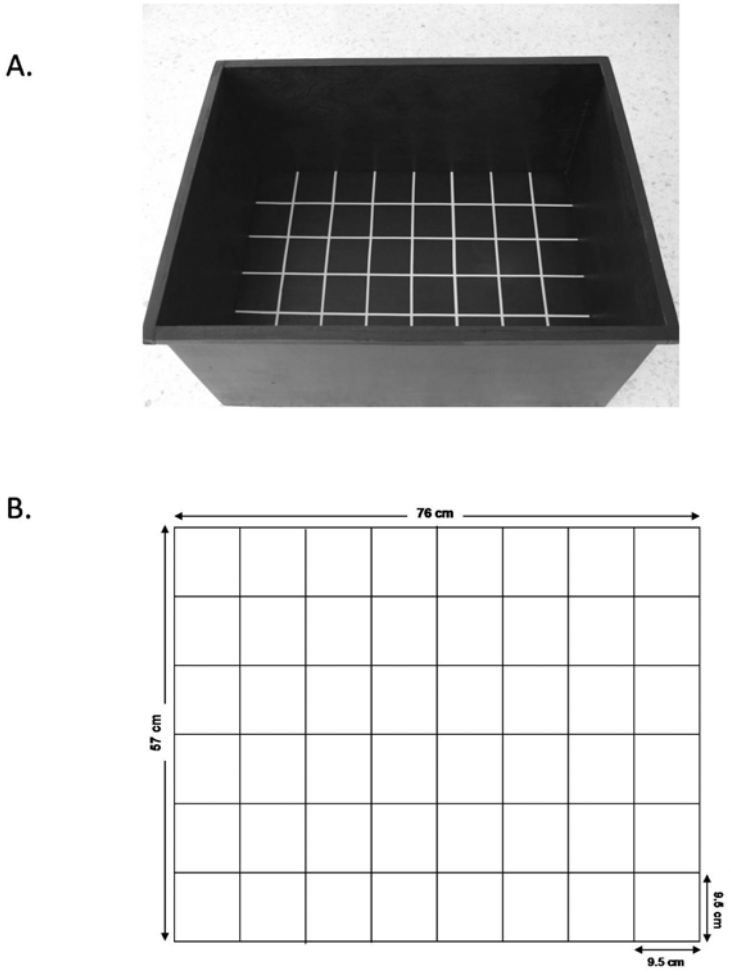


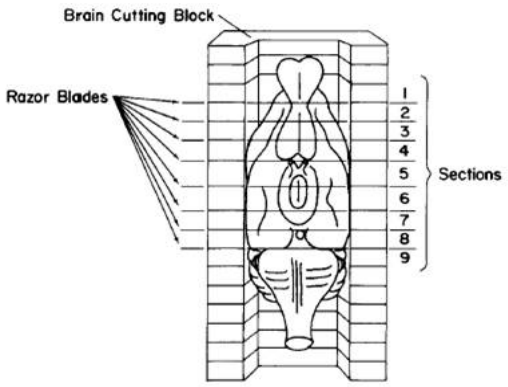
Figure 3-2 The open field (A) was a rectangular box of dimension (57 x 76 x 50 cm), in which the floor (B) was divided into 6 x 8 squares (9.5 x 9.5 cm).

3. Brain dissection

After the open field test, the rats were euthanized and their brains were rapidly removed, frozen in liquid nitrogen and stored at -80 °C. Brains were dissected 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 3-3A). All of the following procedures were 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 sliced 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 seven razor blades were inserted anterior or posterior to the first blade. The brain was thus divided into 8 sections (Figure 3-3A). 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. Then, brain regions were bilaterally dissected from these slices (Figure 3-3B). The amygdala, frontal cortex, hippocampus, nucleus accumbens, septum, anterior hypothalamus and midbrain were collected for HPLC; while the amygdala and midbrain were collected for real-time PCR analysis.

The frontal cortex was consisted of the frontal poles, cortical tissue from section 1, as well as the cortical tissue superior to the rhinal sulcus from sections 2 and 3. The nucleus accumbens was dissected from the rostral surface of the third brain section. The septum was cut from the caudal surface of the fourth brain section based on their distinct morphological appearances. The anterior hypothalamus was collected from the caudal surface of sections 5 and the amygdala included the tissue lateral to both portions of the hypothalamus and ventral to the rhinal sulcus. The hippocampus was separated from the midbrain and overlying cerebral cortex from sections 7 and 8 based on its distinct morphological appearance (Figure 3-3B).

A



B

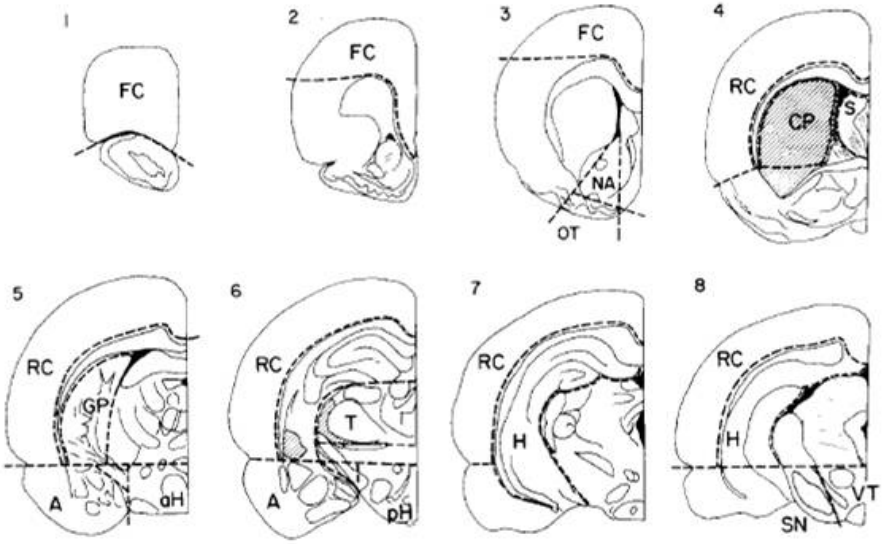


Figure 3-3 **(A)** Diagrammatic representation of brain cutting block illustrating orientation of brain and placement of razor blades to obtain coronal brain sections. The numbers on the right refer to brain sections. **(B)** Diagrammatic representation of coronal brain sections from which brain regions are dissected. 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 A. "Reprinted from Pharmacology Biochemistry and Behavior, 13(3), Heffner T. G., Hartman J. A. and Seiden L. S., A rapid method for the regional dissection of the rat brain, 453-456, copyright 1980, with permission from Elsevier".

4. 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. Then, the samples were centrifuged at 5,000x g (Andreas Hettich universal 32R, Tuttlingen, 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).

4.1 HPLC-analysis

HPLC-EC, a glassy carbon working electrode and an 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 injector (Cotati, CA, U.S.A.), 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, filtered through a 0.22-µm filter, degassed by ultrasonic agitation and pumped at a flow-rate of 0.8 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 lysate 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 chromatogram.

4.2 Analytical procedures

Standard solutions at different concentrations were injected into HPLC system. The retention time was evaluated by injecting the standard 5-HT, 5-HIAA and DHBA individually, and by the injection of a standard mixture. The concentrations of 5-HT and 5-HIAA were calculated by reference to standards and internal standard using peak integration and expressed as ng/mg brain wet weight.

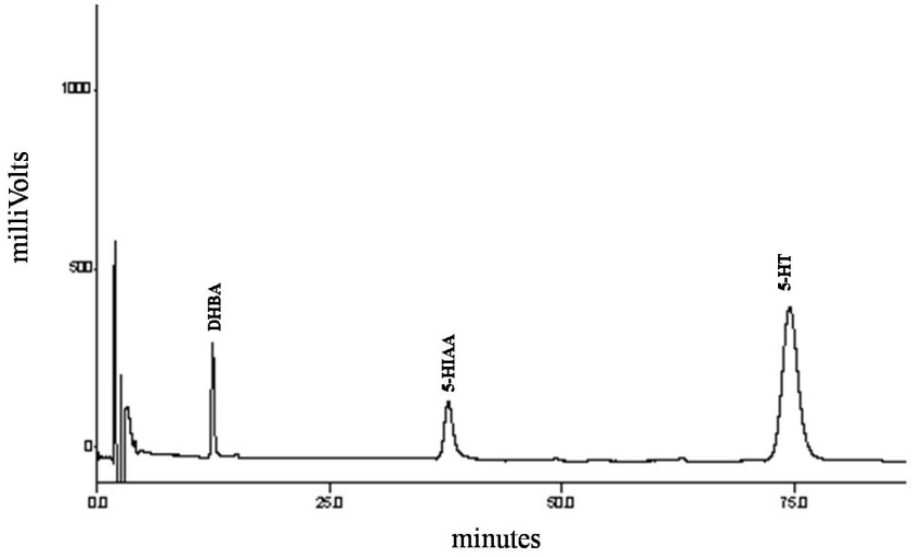


Figure 3-4 The chromatogram represents peaks of standard DHBA, 5-HIAA and 5-HT measured by HPLC-EC. The retention times of DHBA, 5-HIAA and 5-HT were approximately 12.38, 37.75 and 74.41 minutes, respectively.

5. RNA extraction, Reverse Transcription and Real-Time -Polymerase Chain Reaction analysis

5.1 Isolation of RNA

In all groups, midbrain and amygdala were isolated as described in the previous section. The total RNA was extracted from isolated brain tissue with Aurum^{TM} Total RNA Fatty and Fibrous Kit (BioRad) in accordance with the manufacturer's instruction. Briefly, brain tissue approximately 50 mg was transferred into 700 µl of PureZOL RNA solution reagent, immediately disrupted and homogenized by passing through 18, 23 and 26-gauge needle and syringe until homogeneous. Then, it was incubated at room temperature for 5 min to allow complete dissociation of protein complexes. Subsequently, 200 µl of chloroform was added to the lysate, mixed and incubated at room temperature for 5 min. Thereafter, the lysate was centrifuged at 12,000x g (Andreas Hettich universal 32R, Tuttlingen, Germany) for 15 min at 4° C to achieve separation of the organic and aqueous phases.

Then, the aqueous phases, contained the RNAs, were carefully transferred to 1.5 ml microcentrifuge tube, and mixed with 70% ethanol. The sample was then transferred to the Aurum RNA binding mini column, where nucleic acids get bound and centrifuged at 12,000x g for 1 min to discard the supernatant. Wash steps were performed to remove proteins and other cellular debris. Consequently, 80 µl of DNase I was added to the membrane stack of each column and incubated at room temperature for 15 min to remove any remaining genomic DNA and followed by centrifuged at 12,000x g for 1 min to discard the DNase I solution. Then wash steps were performed to removed other debris. Finally, the total RNA was eluted with elution solution. The concentration of total RNA was determined by spectrophotometer with an expected absorbance ratio reading at 260 and 280 nm closed to 2.0, denoting an acceptably pure nucleic acid sample.

5.2 Reverse Transcription

Total RNA (1 µg) was reverse-transcribed into complementary DNA (cDNA) using random-primer in the iScript^{TM} select cDNA Synthesis kit (Bio-Rad). For each 20 µl reverse transcription reaction mixture containing 1 µg of total RNA, 2 µl random-primers and nuclease-free water was incubated at 65˚C for 5 min. Then, 4 µl of 5x iScript selected reaction mix (containing dNTPs, magnesium chloride and stabilizers) and 1 µl of iScript reverse transcriptase enzyme were added and incubated at 25˚C for 5 min followed by 42˚C and 85˚C for 5 min, respectively. The synthesized cDNA was then quantified with spectrophotometer and stored at -20 ˚C for later analysis.

5.3 Real-Time -Polymerase Chain Reaction

The real-time PCR assays were performed with an ABI 7300 instrument (Applied Biosystems, Foster City, California, USA) using the power SYBR® Green master Mix (Applied Biosystems) in accordance with the manufacturer's protocol. For each 25 µl PCR reaction, it composed of 1.5 µg of cDNA, 12.5 µl of SYBR green mastermix, 0.5 µl of each of the forward and reverse primers (20 µM) and 10.5 µl of nuclease free water. All reactions were performed in duplicate. The PCR was performed under the following conditions: 95˚C for 10 min

followed by 40 cycles of denaturation at 95˚C for 30 sec, annealing and extension at 58˚C for 1 min. In each cycle, fluorescent signals were detected at the end of extension stage. To confirm specificity of each product, a dissociation step was performed at the end of PCR, consisting of 95˚C for 15 sec, 60˚C for 30 sec and 95˚C for 15 sec.

The quantification of each mRNA, the calculation was done using the comparative threshold cycle method (Applied Biosystems; User Bulletin No. 2). After performing the PCR, the line whose intersected with the amplification plot was set above the baseline but still in the exponential phase of the semi-log plot; this line was designated as the threshold. The cycle number at which the fluorescence passed the threshold defined as threshold cycle (CT). In the present study, the threshold was set at 0.2 of the semi-log plot. The 18s rRNA, was used as an internal control, against which each target signal was normalized; referred as the ΔCT. The relative expression of the target relative to the internal control was then calculated using the formula $2^{-\Delta CT}$ (Schmittgen and Livak, 2008). Table 3-1 shows the nucleotide sequences for the primers used in this study.

Table 3-1 Gene-specific real-time PCR primers for rat $GABA_A$ receptor subunits and 18s rRNA (internal control)

Genes	Accession No.	Primer (5'-3')	Size (bp)
α2			
sense	L08491	CCAGGATGACGGAACATTGC	78
antisense		GGAAAGTCCTCCAAGTGCATTG	
α3			
sense	L08492	TGGACTCCAGATACCTTCTTCCA	96
antisense		GGTCCCATTGTCTACCAGTCTGA	
α4			
sense	L08493	CACGATGACCACCCTAACGA	66
antisense		ATGGCAGTCGCATAGGACACT	
18s rRNA			
sense	AF102857	CCGCGGTTCTATTTTGTTGGTTTT	399
antisense		CGGGCCGGGTGAGGTTTC	

6. Statistical analysis

All data were presented as means and standard errors of mean (SEM). For comparison between groups, Student's unpaired *t*-test, two-way analysis of variance (ANOVA) or one-way ANOVA followed by the Duncan' multiple comparisons test was used as appropriate. Differences were considered statistically significant at $P < 0.05$.

In experiment 1, the physiological data were analyzed with Student's unpaired *t*-test to make a comparison between groups at each time point. In order to determine the effects of estrogen or length of ovariectomy on anxiety in the ETM and on the changes of GABA or serotonin, the two-way ANOVA with treatment and time of ovariectomy as independent factors was used. In case of significant effects of treatment or time, the Duncan' multiple comparisons test was then used for further analysis. Other data were analyzed with one-way ANOVA followed by Duncan's multiple comparisons test. The correlations between parameters were done with Pearson's correlation.

In experiment 2, in order to determine the effects of estrogen or dosages of benzodiazepine agonist on anxiety in the ETM and on the changes serotonergic system, the two-way ANOVA with treatment and dosages as independent factors was used. In case of significant effects of treatment or dosages, the Dunnett's test was then used for further analysis. Other data were analyzed with one-way ANOVA followed by Dunnett's test.

In experiment 3, all data were analyzed with Student's unpaired *t*-test to make a comparison between groups at each time point or to determine the effects of benzodiazepine on all behavioral and neurochemical data within group.

CHAPTER IV
RESULTS

The results of this study were organized into three parts as follows:

Part 1: To investigate the effects of estrogen deprivation on anxiety-like behaviors, $GABA_A$ receptor subunits gene expression and serotonergic activity in brain associated with anxiety in ovariectomized rats

Part 2: To investigate whether lacking of estrogen causes alteration of $GABA_A$ receptor function and whether these alterations affect serotonergic activity in brain associated with anxiety

Part 3: To investigate whether estrogen can alleviate anxiety-like behavior in ovariectomized rat and whether the $GABA_A$ receptor function is modulated by estrogen, which in turn modulates the serotonergic activity in brain areas related to anxiety