EFFECTS OF ESTROGEN ON $GABA_A$ RECEPTOR PLASTICITY AND ANXIETY-LIKE BEHAVIOR IN OVARIECTOMIZED RATS

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ผลของฮอร์โมนเอสโตรเจนต่อการเปลี่ยนแปลงของตัวรับกาบาเอ และพฤติกรรมวิตกกังวลในหนูแรทที่ถูกตัดรังไข่

นางสาวสุวภรณ์ แดนดี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Thesis Title	EFFECTS OF ESTROGEN ON $GABA_A$ RECEPTOR PLASTICITY AND ANXIETY-LIKE BEHAVIOR IN OVARIECTOMIZED RATS
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สุวภรณ์ แดนดี : ผลของฮอร์โมนเอสโตรเจนต่อการเปลี่ยนแปลงของตัวรับกาบาเอ และพฤติกรรมวิตกกังวลในหนูแรทที่ถูกตัดรังไข่. (EFFECTS OF ESTROGEN ON $GABA_A$ RECEPTOR PLASTICITY AND ANXIETY-LIKE BEHAVIOR IN OVARIECTOMIZED RATS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ.สพ.ญ.ดร.สฤณี กลันทกานนท์ ทองทรง, อ.ที่ปรึกษาวิทยานิพนธ์ ร่วม: รศ.น.สพ.ดร.บุญฤทธิ์ ทองทรง, 114 หน้า.

การศึกษานี้ ศึกษาถึงผลของฮอร์โมนเอสโตรเจนที่มีต่อพฤติกรรมกังวลโดยเน้นการเปลี่ยนแปลงการทำงานของระบบประสาทกาบา และซีโรโทนินในสมองส่วนที่ทำหน้าที่ควบคุมพฤติกรรมวิตกกังวล โดยมีสมมุติฐาน คือ ระยะเวลาของการขาดฮอร์โมนเอสโตรเจนมีผลในเชิงลบต่อพฤติกรรมวิตกกังวลเมื่อทำการทดสอบด้วยอุปกรณ์วัดความกังวล Elevated T-maze (ETM) และผลดังกล่าวอาจเกี่ยวข้องกับการเปลี่ยนแปลงของหน่วยย่อยของตัวรับกาบาเอและ/หรือการทำงานของระบบประสาทซีโรโทนินในสมองส่วนที่เกี่ยวกับภาวะวิตกกังวล เพื่อทดสอบสมมุติฐานดังกล่าว จึงทำการแบ่งการทดลองออกเป็น 3 ตอน ตอนที่ 1 ศึกษาผลของระยะเวลาในการขาดฮอร์โมนเอสโตรเจนต่อภาวะวิตกกังวล และการแสดงออกของตัวรับกาบาเอและระดับสารสื่อประสาทซีโรโทนินในสมอง ในการทดลองแบ่งหนูเพศเมียพันธุ์วิสตาร์ ออกเป็น 2 กลุ่มได้แก่ กลุ่มที่ถูกตัดรังไข่ และกลุ่มที่ตัดรังไข่และได้รับฮอร์โมนเอสโตรเจนทดแทน จากนั้นสุ่มหนูในแต่ละกลุ่มเพื่อทดสอบด้วย ETM ในวันที่ 7 14 21 หรือ 28 ภายหลังจากการตัดรังไข่ พบว่าหนูที่ถูกตัดรังไข่เป็นเวลา 21 และ 28 วัน มีระดับความกังวลสูงขึ้นเมื่อเทียบกับหนูที่ถูกตัดรังไข่ 7 และ 14 วัน โดยระยะเวลาที่ขาดฮอร์โมนเอสโตรเจนมีความสัมพันธ์ในเชิงลบกับระดับของความวิตกกังวล สำหรับหนูกลุ่มที่ตัดรังไข่และได้รับเอสโตรเจนทดแทนไม่แสดงพฤติกรรมกังวล เมื่อทำการวัดระดับสารสื่อประสาทซีโรโทนินและการแสดงออกของยีนหน่วยย่อยของตัวรับกาบาเอ พบว่าระบบประสาทซีโรโทนินในหนูที่ถูกตัดรังไข่มีการเปลี่ยนแปลงไม่แน่นอนเมื่อเทียบกับหนูที่ได้รับฮอร์โมนเอสโตรเจน สำหรับการแสดงออกของยีนหน่วยย่อยของตัวรับกาบาเอ พบว่าการแสดงออกของยีนหน่วยย่อยของตัวรับกาบาเอชนิด $\alpha 2$ $\alpha 3$ และ $\alpha 4$ ในสมองส่วนกลางในหนูที่ถูกตัดรังไข่สูงกว่าหนูที่ได้รับฮอร์โมนเอสโตรเจนโดยเฉพาะชนิด $\alpha 2$ และ $\alpha 3$ และเป็นที่น่าสังเกตคือยีนดังกล่าวมีการแสดงออกเพิ่มขึ้นในวันที่ 21 ซึ่งสอดคล้องกับพฤติกรรม สำหรับหนูที่ได้รับฮอร์โมนเอสโตรเจน การแสดงออกของยีนหน่วยย่อยของตัวรับกาบาเอมีระดับค่อนข้างคงที่ ตอนที่ 2 เพื่อศึกษาการทำงานของตัวรับกาบาเอในหนูที่ถูกตัดรังไข่ และได้หรือไม่ได้รับฮอร์โมนเอสโตรเจน เป็นระยะเวลา 3 สัปดาห์ (ซึ่งเป็นระยะที่พบการเปลี่ยนแปลงพฤติกรรม) โดยทำการทดลองเช่นเดียวกับตอนที่ 1 แต่ทำการฉีด benzodiazepine agonist (diazepam ขนาด 0 0.25 0.5 หรือ 1 มก/กก.) 30 นาทีก่อนทดสอบพฤติกรรม ผลการทดลองพบว่า ตัวรับกาบาเอมีความไวเพิ่มขึ้นในหนูที่ถูกตัดรังไข่ ตอนที่ 3 เพื่อศึกษาผลของฮอร์โมนเอสโตรเจนในการรักษาภาวะวิตกกังวล และการเปลี่ยนแปลงการทำงานของตัวรับกาบาเอ ในการทดลองนี้หนูถูกตัดรังไข่เป็นเวลา 3 สัปดาห์ เพื่อประกันการขาดฮอร์โมนเอสโตรเจน ก่อนนำมาแบ่งเป็น 2 กลุ่ม คือกลุ่มที่ได้ หรือไม่ได้ฮอร์โมนเอสโตรเจนทดแทน เป็นเวลา 4 สัปดาห์ ในวันทดสอบพฤติกรรมหนูในแต่ละกลุ่มถูกแบ่งเป็น 2 กลุ่มย่อย คือ ได้หรือไม่ได้รับ diazepam ขนาด 0.25 มก/กก (ซึ่งเป็นขนาดที่ให้ผลในการทดลองที่ 2) เพื่อทดสอบการทำงานของตัวรับกาบาเอ ผลการทดสอบชี้ให้เห็นว่าเอสโตรเจนสามารถแก้ไขภาวะวิตกกังวลได้ โดยส่วนหนึ่งน่าจะเป็นผลจากการทำงานของตัวรับกาบาเอ สำหรับการทำงานของระบบประสาทซีโรโทนินที่เกิดจากการทำงานของตัวรับกาบาเอ (ภายหลังจากให้ diazepam) ในการทดลองที่ 2 และ 3 พบว่าระดับสารสื่อประสาทซีโรโทนิน เมตาบอไลท์ และสัดส่วนของเมตาบอไลท์ต่อสารสื่อประสาทซีโรโทนินในสมองทุกส่วนที่วัดในหนูที่ถูกตัดรังไข่ไม่แตกต่างกัน ในขณะที่หนูที่ได้รับฮอร์โมนเอสโตรเจน มีการเพิ่มขึ้นของระดับสารสื่อประสาทซีโรโทนินในสมองบางส่วน จากการทดลองสรุปได้ว่าระยะเวลาในการขาดฮอร์โมนเอสโตรเจนที่เพิ่มขึ้นมีผลให้ระดับความกังวลเพิ่มสูงขึ้น และการเปลี่ยนแปลงนั้นส่วนหนึ่งเป็นผลจากการทำงานของตัวรับกาบาเอที่เปลี่ยนแปลงไป จากการเพิ่มขึ้นหรือลดลงของการแสดงออกของยีนหน่วยย่อยของตัวรับกาบาเอ

สาขาวิชา……สรีรวิทยา…..... ลายมือชื่อนิสิต …….....

ปีการศึกษา…….2554……..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก…….....

ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์ร่วม…….....

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KEYWORDS: ANXIETY / BENZODIAZEPINE / ESTROGEN / $GABA_A$ RECEPTOR / OVARIECTOMIZED RAT

SUWAPORN DAENDEE: EFFECTS OF ESTROGEN ON $GABA_A$ RECEPTOR PLASTICITY AND ANXIETY-LIKE BEHAVIOR IN OVARIECTOMIZED RATS.ADVISOR: ASST.PROF.SARINEE KALANDAKANOND THONGSONG, D.V.M., Ph.D., CO-ADVISOR: ASSOC.PROF. BOONRIT THONGSONG, D.V.M., Ph.D., 114 pp.

In the present study, the effects of estrogen on anxiety-like behavior and the alterations of GABAergic and serotonergic systems were focused. It was hypothesized that the length of estrogen deprivation had a negative effect on the level of anxiety-like behavior measured with the elevated T-maze (ETM); and this effect may be related to the alteration of $GABA_A$ receptor subunits and/or serotonergic activity in the brain areas associated with anxiety. In order to test these hypotheses, this study was divided into 3 parts. In part 1, to determine the length of estrogen deprivation on anxiety behavior and the $GABA_A$ receptor subunit mRNA expressions along with the changes in serotonin levels, the female Wistar rats were divided into 2 groups, ovariectomized-rat (Ovx) and ovariectomized-rat with estrogen replacement (E_2). Then the rats from each group were randomly selected for behavioral test at 7, 14, 21 or 28 days after ovariectomy,. The behavioral data from the ETM demonstrated that the rats that were deprived of estrogen for 21 and 28 days had higher level of anxiety compared to those at day 7 and 14. Moreover, a significant negative correlation between the time of estrogen deprivation and the level of anxiety was found. For the E_2 groups, the number of day following ovariectomy had no significant effect on anxiety behavior. After behavioral tests, the measurement serotonin (5-HT) and its metabolite (5-HIAA), and $GABA_A$ receptor subunit gene expression were examined. In conjunction to the behavioral data, the serotonergic activity was more fluctuated in the Ovx rats compared to the E_2 rats. For $GABA_A$ receptor subunit gene expression, the α2-, α3- and α4 $GABA_A$ receptor subunit gene expressions in the midbrain were higher in the Ovx than the E_2 groups especially for α2- and α3-$GABA_A$ receptor subunits. Interestingly, the α3- and α4- receptor subunits were markedly up-regulated at day 21 in the Ovx groups. Contrarily, the expression levels in the E_2 groups were rather stable. These results suggested the alteration in GABAergic and serotonergic systems in relation to behavior. In part 2, the $GABA_A$ receptor function was determined in the 3 weeks-Ovx rats with or without E_2 supplementation by injecting benzodiazepine agonist (diazepam, 0, 0.25, 0.5 and 1 mg/kg) 30 min before behavioral test. The results in this part indicated that the $GABA_A$ receptor sensitivity was increased in the 3 week ovariectomized rats. In part 3, to determine the effect of estrogen in treating anxiety along with the alteration of $GABA_A$ receptor function; the rats were first ovariectomized for 3 weeks to warrant the estrogen depletion, before supplemented with or without E_2 for 4 weeks. On the behavioral test day, the rats from each group were subdivided into 2 groups receiving vehicle or diazepam (0.25 mg/kg; the effective dose from part 2) in order to test the function of $GABA_A$ receptor. In this part, the data indicated that estrogen could alleviate anxiety in anxious rats. For the serotonergic activity following diazepam administration as in parts 2 and 3, there was no difference in the levels of 5-HT and 5-HIAA or the ratio of 5-HIAA/5-HT in the Ovx groups in all examined brain areas; whereas, in the E_2 groups, the 5-HT levels was significant increased in some areas. Therefore, this information contributes to the roles of estrogen in generating anxiety in relation to the GABAergic system.

Field of Study: .....Physiology..... Student's Signature.....

Academic Year:.....2011..... Advisor's Signature.....

Co-advisor's Signature.....

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LIST OF ABBREVIATIONS

°C	degree Celsius
µg	microgram
µl	microliter
µm	micrometer
µM	micromolar
5-HIAA	5-hydroxyindoleacetic acid
5-HT	serotonin
5-HTP	5-hydroxytryptophan
8-OH-DPAT	8-hydroxy-2-(di-N-propylamino) tetralin
A	amygdala;
AADC	aromatic L-amino acid decarboxylase
ACh	acetylcholine
Ag	chemical symbol; Silver
AgCl	silver chloride
aH	anterior hypothalamus
am	ante meridiem
BDZs	benzodiazepines
bp	base pair
BW	body weight
cDNA	complementary deoxyribonucleic acid
cm	centimeter
CP	caudate putamen
CT	threshold cycle
d	day
DA	dopamine
DFI	daily food intake
DHBA	3,4-dihydroxy-benzyl-amine hydrobromide
DNase I	deoxyribonuclease I
DSM	diagnostic and statistical manual
DWG	daily weight gain

E2	17β-estradiol
EC_{50}	effective concentration
EPM	elevated plus-maze
ER	estrogen receptor
ERT	estrogen replacement therapy
ETM	elevated T-maze
FC	frontal cortex
GABA	gamma-aminobutyric acid
GAD	glutamic acid decarboxylase
gm	gram
GP	globus pallidus
H	hippocampus
HPA	hypothalamic-pituitary-axis
HPLC-EC	high-performance liquid chromatography - electrochemical detection
hr	hour
i.p.	intraperitoneal injection
kg	kilogram
lx	lux
M	molarity
MAOI	monoamine oxidase inhibitors
MDMA	3,4-methylenedioxymethamphetamine
mg	milligram
min	minute
ml	milliliter
mM	millimolar
mRNA	messenger RNA
n	number
nA	nano ampere
NA	nucleus accumbens
NE	norepinephrine
ng	nanogram

nm	nanometer
No.	number
NPY	neuropeptide Y
OCD	obsessive-compulsive disorder
OT	olfactory tubercle
Ovx	ovariectomized
PAG	periaqueductal gray
PCR	polymerase chain reaction
PD	panic disorder
pH	posterior hypothalamus
pm	post Meridiem
PTSD	post-traumatic stress disorder
RC	remaining cortex
RNA	ribonucleic acid
rRNA	ribosomal RNA
s	seconds
S	septum
SEM	standard errors of mean
SN	substantia nigra
SSRI	selective serotonin reuptake inhibitor
T	thalamus
TCAs	tricyclic antidepressants
UW	uterine weight
V	volt
veh	vehicle
VT	ventral tegmentum

CHAPTER I
INTRODUCTION

Anxiety is one of the most common psychological symptoms found in people, approximately 28.8 % of the populations are anxiety disorders and the incidence of clinical anxiety is increasing around the world (Kessler et al., 2005; Wittchen et al., 2011). Interestingly, women were more suffering from anxiety disorder than men (Seeman, 1997; Lim et al., 2005) especially during low estrogen levels i.e. following oophorectomy or during postmenopausal period (Avis et al., 2001; Schmidt, 2005). Such women required estrogen replacement therapy to improve these psychological symptoms (Ditkoff et al., 1991; Schmidt et al., 2000; Zeidan et al., 2011). These findings indicated that the ovarian steroid hormones especially estrogen may be partially responsible for anxiety disorder (Weiser et al., 2008; Hiroi and Neumaier, 2011). However, the molecular mechanism of estrogen to modulate anxiety is not as simple and possibly involved the different effects of estrogen on enzyme and neurotransmitter functions in brain related to anxiety.

The neural circuits that underlie anxiety were organized at different levels. Electrical and pharmacological studies indicated that the midbrain plays a key role in regulating anxiety (Schenberg et al., 2001; Zanoveli et al., 2005). In human and animal studies, anxiety-like responses such as unpleasant and fear-like sensations or freezing behavior were found when midbrain was stimulated (Amano et al., 1978; Schenberg et al., 2001; Brandao et al., 2003). The midbrain is known to contain a population of serotonergic neuron and project axon to innervate the limbic system, which is critically involved in regulation of anxiety (Beitz et al., 1986; Clement and Chapouthier, 1998; Oliveira et al., 2004). Activation of the serotonergic neuronal function could facilitate the generation of anxiety, whereas reduction of serotonergic function could induce the anxiolytic effects (Matos et al., 1996; Clement and Chapouthier, 1998). Moreover, Pandaranandaka and co-workers (2006; 2009) have shown that the alteration of serotonergic activity was related to anxiety disorder. In addition, the first-line prescribed drugs for treating anxiety is likely to modulate serotonergic system at some points; for example, selective serotonin reuptake

inhibitor (SSRI), 5-HT_{1A} agonist and monoamine oxidase inhibitor (Nash and Nutt, 2005; Koen and Stein, 2011).

Although various neurotransmitter systems are involved in the pathogenesis of anxiety disorder, the GABAergic system may be of major concerned as indicated by previous evidences (Carey et al., 1992; Malizia et al., 1998; Reddy and Kulkarni, 1999). Firstly, the most widely prescribed anxiolytic drugs are benzodiazepine groups which are acting on the $GABA_A$ receptor (Lopez-Munoz et al., 2011). Secondly, several anatomical studies found that serotonergic neurons in midbrain were under GABAergic system control (Gervasoni et al., 2000; Tao and Auerbach, 2000; Castilho et al., 2002). Local applications of $GABA_A$ receptor agonist, muscimol, into the midbrain inhibited serotonergic activity in the midbrain and forebrain, which could be blocked by $GABA_A$ receptor antagonist, bicuculline (Tao and Auerbach, 1994; Tao et al., 1996; Li et al., 2005). These results indicated that $GABA_A$ receptors in the midbrain have an important role in the regulation of serotonergic activity and thus dysfunction of $GABA_A$ receptor may contribute to the alteration of serotonergic system and lead to anxiety disorder. Finally, previous studies showed that the decrease in $GABA_A$ receptor sensitivity was found in anxiety disorder patient as demonstrated from [^{14}C] flumazenil positon emission tomography (Malizia et al., 1998; Abadie et al., 1999; Hasler et al., 2009). These altogether indicated that alteration of $GABA_A$ receptor function probably affected anxiety disorder.

There are number of studies indicated that ovarian steroid hormone can modulate $GABA_A$ receptor function (Henderson and Jorge, 2004; Picazo et al., 2006). For example, the sensitivity to benzodiazepine or GABA had been reported to be changed across the estrous cycle in rodents (Carey et al., 1992; Reddy and Kulkarni, 1999). Female mice were more responsive to the anxiolytic effects of diazepam during estrus and diestrus, but no response during proestrus or metestrus phase (Carey et al., 1992). In ovariectomized rat, the sensitivity of benzodiazepine was varied with the length of ovariectomy (Picazo et al., 2006). Interestingly, *in vitro* and *in vivo* studies had shown that estrogen can alter the mRNA expression of $GABA_A$ receptor subunit (Herbison and Fenelon, 1995; Gulinello et al., 2003; Pierson et al., 2005; Byrnes et al., 2007; Henderson, 2007). Herein, the deficiency of estrogen may affect

gene expression of GABA receptor subunits in brain and lead to the functional alteration of $GABA_A$ receptor, which has been postulated to the changing of behavior and emotion. However, few studies investigated the estrogenic effect on the plasticity of the $GABA_A$ receptor subunit gene expression in brain areas related to anxiety in animal models.

Therefore, the objectives of present study were as follows:

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

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

3) To investigate whether estrogen can alleviate anxiety-like behavior in ovariectomized-induced anxiety rat, in relation to the recovery of $GABA_A$ receptor function and the serotonergic activity in brain areas related to anxiety

This study would provide more information concerning the roles of estrogen in causing, preventing and treating anxiety which can be used as supportive information for the therapeutic effect of estrogen on anxiety disorders.

CHAPTER II
LITERATURE REVIEWS

A. Anxiety

Anxiety is a normal emotional response to various stressors and then subsides when the stressors diminishes (Clement and Chapouthier, 1998). Generally, when anxiety occurs, mood and cognitions may alter such as lost of intention or cognitive impairment and associated with physiological or behavioral changes such as palpitations, irritability and hypervigilance (Vanin, 2008). However, anxiety is considered to be pathological when excessive and persistent. According to the fourth edition of the Diagnostic and Statistical Manual (DSM-IV) of the American Psychiatric Association, anxiety disorder can be classified into panic disorder (with or without agoraphobia), agoraphobia, social phobia, obsessive-compulsive disorder, post-traumatic stress disorder and generalized anxiety disorder.

1) Panic disorder is characterized by feelings of extreme fear and accompanied by physiological symptoms such as irritability, tachycardia and dizziness. Patient is diagnosed when multiple panic attacks or one panic attack and followed by persistent fear of having further attack (Graeff and Del-Ben, 2008).

2) Agoraphobia is characterized by feeling fear of open spaces or situation that the patient feels there was no escape from this space or situation (Shelton and Hunt, 2008).

3) Social phobia is an excessive fear or more anxiety while being exposing to social situation (such as public speaking or unfamiliar people), resulting in impairments in social and work functioning, or create significant distress (Kaminer and Stein, 2003).

4) Obsessive-compulsive disorder is diagnosed when patients experience incessantly obsessive thoughts and perform repetitive, compulsive acts aimed at alleviating these thoughts and anxiety they produce (Kandel, 2000; Shelton and Hunt, 2008).

5) Post-traumatic stress disorder is occurred following extremely stressful event, such as physical abuse or life-threatening combat (Kandel, 2000; Shelton and Hunt, 2008).

6) Generalized anxiety disorder is characterized by excessive worry and is prolonged more than six months. The symptoms are motor tension, autonomic hyperactivity and vigilance (Tyrer and Baldwin, 2006). This form of anxiety is the most common form of anxiety with an estimated population prevalence of 3% (reviewed by Lenze and Wetherell, 2011; Hickey et al., 2012)

Much information has shown that various neurotransmitters or neuromodulator systems, such as serotonergic, norepinephrinergic, dopaminergic and GABAergic systems, were involved in anxiety (Graeff et al., 1996; Weinberger, 2001; Millan, 2003; Gordon and Hen, 2004). Investigations into neurotransmitter dysfunction have implicated the involvement of serotonergic and GABAergic systems in panic and generalized anxiety disorder (Tork, 1990; Wu et al., 1997; Guimaraes et al., 2010; Mohler, 2012). Moreover, the common and widely used anxiolytic drugs are selective serotonin reuptake inhibitors (SSRIs), antidepressants drugs such as tricyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAOIs) and benzodiazepine groups (Nash and Nutt, 2005; Koen and Stein, 2011). The SSRIs and TCAs have slow action and only effective to some types of anxiety disorder (Argyropoulos et al., 2000). On the contrary, benzodiazepine groups have very fast action, directly block acute anxiety disorder and are effective to treat all forms of anxiety disorders (Argyropoulos et al., 2000). These results indicated that GABAergic system may play an important role in all types of anxiety disorder.

B. Neural Circuitry of Anxiety

The neural circuits that underlie anxiety were organized at different levels. It is well known that the limbic system is a major center in the genesis of emotions, especially anxiety. The limbic system interacts with other brain areas including midbrain to influence emotional tone. In general, the afferent neurons from midbrain project axon to innervate the limbic system including amygdala, hippocampus and frontal cortex, which are critically involved in the regulation of anxiety (Oliveira et al., 2004). Then, the efferent neurons from the amygdala innervate the periaqueductal

gray, brainstem, midbrain and hypothalamus, which initiate fear-related behavioral, autonomic and hormonal responses (Davis et al., 1994; LeDoux, 2000). The axons project from the central nucleus of the amygdala to the hypothalamus appeared to be involved in activation of the sympathetic autonomic nervous system during fear or anxiety (Davis et al., 1994; LeDoux, 2000). The axons project from the amygdala to the ventral tegmental area increased dopaminergic activity in the prefrontal cortex (Millan, 2003). In addition, neurons project from the amygdala to locus coeruleus or raphe neuron could activate norepinephrine or serotonin released and leads to enhance motor performance (Millan, 2003). In humans, stimulation of midbrain produces unpleasant and fear-like sensations that resemble the symptomatic expression of panic attack (Amano et al., 1978). In animal study, either chemical or electrical stimulation of this structure induces freezing behavior alternating with vigorous flight (Jung et al., 2001; Schenberg et al., 2001). This behavior has also been identified as panic-like behavior (Schenberg et al, 2001; Brandao et al., 2003). Therefore, the normal function of this circuit is critical for physiological anxiety, while dysfunction of this circuit will lead to pathological anxiety.

Anatomical investigations have revealed that the serotonergic cell bodies are clustered in the brainstem and midbrain (Frazer and Hensler, 1999). The majority of serotonergic cell bodies are found mainly in raphe nuclei, which was divided into dorsal and median raphe nuclei (Frazer and Hensler, 1999). The serotonergic neuron originating in the dorsal raphe projection axons innervates the frontal cortex, hippocampus, and amygdala. While the ascending neuron from the median raphe project to innervate the hippocampus, hypothalamus, nucleus accumbens and septum, (Millan, 2003; Guimaraes et al., 2010). There was some information indicating that the abnormalities of the serotonergic pathways had been related to anxiety disorders (Graeff et al., 1996; Clement and Chapouthier, 1998; Graeff and Del-Ben, 2008). For example, increased endogenous 5-HT levels in limbic forebrain structure facilitated anxiety as shown by the direct injection of 5-HT or SSRI into the dorsal periaqueductal gray or basolateral amygdala induced anxiety, while the reduction of 5-HT by the 5-HT_{1A} receptor agonist, 8-OH-DPAT reduced anxiety (Zanoveli et al., 2003; Martinez et al., 2007; Zanoveli et al., 2009). These findings indicated that serotonergic system plays an important role in the pathophysiology of anxiety.

In addition, there was an information indicating that output neuron in midbrain was under GABAergic system control (Judge et al., 2004; reviewed by Adell et al., 2010). Previous studies demonstrated that the serotonergic neuron in the midbrain made a synaptic connection with GABAergic neuron (Harandi et al., 1987; Wang et al., 1992). In addition, there are information indicated that the $GABA_A$ and $GABA_B$ receptors were localized on the serotonergic neuron (Gao et al., 1993; Bischoff et al., 1999; Wirtshafter and Sheppard, 2001; Serrats et al., 2003). The $GABA_A$ receptor was found on the serotonergic neuron (Gao et al., 1993), while the $GABA_B$ receptor was found on both serotonergic neuron and GABAergic interneuron (Wirtshafter and Sheppard, 2001; Serrats et al., 2003). In term of function, the inhibitory effect of GABA on the firing of serotonergic neuron was well established (Judge et al., 2004; reviewed by Adell et al., 2010). For example, local infusion of muscimol, $GABA_A$ receptor agonist into the midbrain raphe nuclei reduced the serotonin tone in forebrain regions including hippocampus and frontal cortex (Tao et al., 1996; Li et al., 2005); whereas bicucullin, the $GABA_A$ antagonist increased serotonin levels (Tao et al., 1996; Tao and Auerbach, 2003). These indicated that $GABA_A$ receptors were tonically active in midbrain (Tao et al., 1996; Li et al., 2005). Furthermore, activation of $GABA_A$ and benzodiazepine receptors within midbrain area inhibited the escape behavior evoked by the electrical stimulation (Castilho et al., 2002; Bueno et al., 2005). These results indicated that the $GABA_A$-benzodiazepine receptors in midbrain may be involved in regulation of anxiety-like behavior.

C. GABAergic system

Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmission in the brain (Olsen and DeLorey, 1999). The GABA is synthesized from glutamate by glutamic acid decarboxylase. The glutamate is converted from glutamine by glutaminase. The glutamine in the GABAergic neuron is transported into the GABAergic neuron by glutamine transporter. The glutamine in the glial cell is derived from glutamate by glutamate synthase. The glutamate in glial cell comes from 2 main sources; from Kreb's cycle and from the GABAergic or glutamatergic neuron. GABA releases into the synaptic cleft and diffuses across the cleft to the

target receptors on the postsynaptic surface. The action of GABA at the synapse is terminated by reuptake into both presynaptic nerve terminals and surrounding glial cells (Deutch and Roth, 2003).

For the effect of GABAergic system on anxiety, many studies supported the view that GABA had a crucial role in the regulation of anxious states by exerted its inhibitory effect on the function of other neurotransmitters associated with increase anxiety such as norepinephrinergic and serotonergic system (Stutzmann and LeDoux, 1999; Shekhar et al., 2002). Local application of $GABA_A$ receptor antagonist elevated the levels of norepinephrine in the hypothalamus (Shekhar et al., 2002). Similarly, $GABA_A$ receptor antagonist also induced an increase in the 5-HT levels in the midbrain and frontal cortex and associated with anxiety (Tao et al., 1996; Li et al., 2005; Lowry and Hale, 2010). Moreover, mice lacking glutamate decarboxylase showed increase anxiety (Stork et al., 2000). While, the reduction in anxiety was demonstrated in rats received drug that increase the levels of GABA such as GABA transporter inhibitor (Schmitt et al., 2002). These evident indicated the important of GABAergic system in regulating anxiety.

For the function of GABAergic system, GABA exerts its inhibitory effect through at least two receptor subtypes, $GABA_A$ and $GABA_B$ receptors. $GABA_A$ receptor is ligand-gated ion channel. It is the major inhibitory receptor in the brain and has the binding sites for many clinically important drugs (Mohler et al., 2001; Mohler, 2012). This type of receptor is believed to be involved in mediating sedative, anticonvulsant, muscle relaxant, amnesic and anxiolytic activity. $GABA_B$ receptor is G-protein coupled receptor. It acts as an autoreceptor to inhibit further GABA release from GABAergic neuron (Deutch and Roth, 2003). However, recent evidence indicated that the $GABA_A$ receptor has an important role in the etiology of anxiety disorder. Therefore, the function of $GABA_A$ receptor was focused in the present study.

$GABA_A$ receptor

The rapid inhibitory action of GABA is mediated via $GABA_A$ receptor which is ligand-gated ion channel that mediates fast synaptic inhibition throughout the brain

(Mohler et al., 2001; Whiting, 2003). The inhibitory action of GABA occurs when GABA binds to $GABA_A$ receptor and leads to the chloride (Cl^-) channel opening, resulting in hyperpolarization of the neuronal membrane and consequent reduction in neuronal activity (Olsen and Macdonald, 2002). The $GABA_A$ receptors not only have GABA binding site but also have various binding sites for several ligands such as benzodiazepine, barbiturates, neurosteroid and ethanol (Mohler et al., 2001). These are allosteric modulators which increased either GABA affinity or frequency of $GABA_A$ receptor opening (Olsen and Macdonald, 2002). Therefore, the $GABA_A$ receptor has an important role in mediating sedative, anticonvulsant, muscle-relaxant, amnestic- and anxiolytic activity (Mohler et al., 2001).

$GABA_A$ receptor is composed of pentamers containing subunits including α1-6, β1-3, γ1-3, δ, ε, θ and π, but the majority of the receptors presented in the central nervous system is composed of α, β and γ subunits (Mohler et al., 2001; Whiting, 2003). The subunit composition of a receptor is an important determinant of its functional properties. For example, combination of α and β subunits is necessary to form an active GABA binding site (Mohler et al., 2001; Whiting, 2003). For benzodiazepines binding site, α and γ subunits are required. Benzodiazepines can bind to the $GABA_A$ receptor only if the α subunits are type 1, 2, 3 or 5; but they cannot bind or respond to benzodiazepine if the α subunits are type 4 or 6. However, barbiturates and neurosteroids seem to have little subtype specificity (Liberzon et al., 2003). Furthermore, the distribution of $GABA_A$ receptor subunits within the brain is region specific (Table 2-1) (Olsen and DeLorey, 1999; Pirker et al., 2000; Mohler et al., 2002). There are information indicated that $GABA_A$ receptor containing α2-subunit is found predominantly in the limbic system and α3 subunit is found mainly on the cholinergic and monoaminergic neurons in the midbrain (Olsen and DeLorey, 1999; Dias et al., 2005). In contrast, $GABA_A$ receptors containing α4- subunits is found predominantly at extrasynaptic locations and is involved in tonic inhibition (Olsen and DeLorey, 1999; Stell et al., 2003). More than 60% of $GABA_A$ receptor in the brain contain α1- subunit which implicated in the sedative effects of benzodiazepine, whereas the α2 and α3 is found about 10-15 % and implicated in the anxiolytic effects of benzodiazepine (Low et al., 2000; Rowlett et al., 2005; Morris et al., 2006). The $GABA_A$ receptor contains α4-, α5-, and α6- subunits is found less than

5% in the brain (Rudolph and Antkowiak, 2004). Thus the actions of GABA are likely to differ depending on receptor subtypes and in different parts of the brain.

Table 2-1 Distribution and pharmacological characteristics of the $GABA_A$ receptor subtype in the rat brain.

Isoform	Relative abundance	Pharmacological Characteristics	Location
α1β2γ2	60%	Mediates the sedative, amnestic, anticonvulsant action of benzodiazepine. High affinity for classical benzodiazepines, zolpidem and the antagonist flumazenil.	Cerebral cortex, hippocampus, dentate gyrus, pallidum, striatum, thalamus, olfactory bulb, cerebellum.
α2β3γ2	15-20%	Mediates anxiolytic action of benzodiazepine. High affinity for classical benzodiazepine agonists and the antagonist, flumazenil. Intermediate affinity for zolpidem.	Cerebral cortex, hippocampus, dentate gyrus, olfactory bulb, striatum, inferior olivary neuron.
α3βnγ2	10-15%	High affinity for classical benzodiazepine agonists and the antagonist, flumazenil. Intermediate affinity for zolpidem.	Cerebral cortex, hippocampus, Brainstem and midbrain (noradrenergic and serotonergic neurons), basal forebrain (cholinergic neurons), thalamus
α4βnγ/ α4βnγδ	< 5%	Insensitive to classical benzodiazepine agonist and zolpidem	Dentate gyrus
α5β1/3γ2	< 5%	High affinity for classical benzodiazepine agonist and the antagonist flumazenil. Very low affinity for zolpidem.	Spinal trigeminal nucleus, superior olivary neurons, cerebral cortex, hippocampus, olfactory bulb
α6β2/3δ	<5%	Insensitive to classical benzodiazepine agonist and zolpidem	Cerebellum
α6βnδ	Minor population	Lacks benzodiazepine site	Cerebellum
ρ	Homomeric receptor	Insensitive bicucullin, barbiturate, balcofen and all benzodiazepine site ligand	Retina

Modified from Olsen and DeLorey, 1999; Mohler et al., 2002.

$GABA_A$ receptor and Anxiety

The evidence from experimental and clinical studies supports an involvement of the GABAergic system and its receptor, especially $GABA_A$ receptors in anxiety disorder (Carey et al., 1992; Malizia et al., 1998; Reddy and Kulkarni, 1999). Firstly, the most widely prescribed anxiolytic drugs were benzodiazepine groups which were acting on the $GABA_A$ receptor (Vanin, 2008); and secondly, previous studies showed that the decrease in $GABA_A$ receptor sensitivity was found in anxiety disorder patient as demonstrated from [^{14}C] flumazenil positon emission tomography (Malizia et al., 1998; Abadie et al., 1999; Hasler et al., 2008). These altogether indicated that alterations of $GABA_A$ receptor function probably cause anxiety disorder.

The evidence for $GABA_A$ receptor involvement in anxiety disorder is supported by using antagonists to block $GABA_A$ receptors which lead to increase in anxiety in both human and animals (File et al., 1984; Gentil et al., 1990); whereas, the agonists that increase GABA function reduce anxiety (Nutt et al., 1990; Jardim et al., 2005). Moreover, the increase in anxiety-like behavior was associated with ontogenetic or phylogenic alterations in receptor numbers or subtypes (Rago et al., 1988; Primus et al., 1992; Dixon et al., 2008). Single-photon emission computed tomography studies have shown that patients with generalized or panic disorder have lowered benzodiazepine receptor binding sites (Malizia et al., 1998) and the sensitivity of $GABA_A$-benzodiazepine receptor also reduced (Roy-Byrne et al., 1996). Similarly, mice with α2 subunit point-mutation were insensitive to diazepam, benzodiazepine agonist and resulting in loss of anxiolytic effects of diazepam (Low et al., 2000; Morris et al., 2006; Dixon et al., 2008). Moreover, pharmacological studies indicated that α3-containing $GABA_A$ receptor also mediated anxiolytic effects (Dias et al., 2005; Morris et al., 2006). These results indicated that impaired $GABA_A$ receptor function probably caused anxiety disorder. Therefore, the reduction of $GABA_A$ receptor expression and/or benzodiazepine binding sites may lead to increase anxiety.

D. Estrogen

Estrogens are steroid hormone, mainly synthesized in the ovaries using cholesterol as a precursor. In human, the most potent estrogen is 17β-estradiol. In

addition to synthesize in ovaries, estrogens can be synthesized locally in the brain by converting androgens to estrogens by an aromatase enzyme and these local synthesis estrogens have been shown to play a major role in synaptogenesis and neurogenesis during development (Naftolin et al., 1988).

Estrogen exerts its effect by genomic and non genomic mechanisms. For genomic mechanism, estrogen binds to intracellular receptor to modulate transcription and translation (McEwen and Alves, 1999). In addition, estrogen can also affect transcription by interacting with AP-1, CREB or other transcription factor families and in turn altering the expression of genes controlled by those transcription factors (McEwen and Alves, 1999). These mechanisms have a response time of several minutes, hours or days. For the non genomic mechanisms, estrogen produces rapid effects (second to minutes) by changing the neuronal activity, which may be due to the changes in G-protein receptor-couple transduction or MAPKs or tyrosine kinase cascades (Simoncini and Genazzani, 2003; Boulware et al., 2005). However, the non genomic action of estrogen in term of intracellular cascade is still unclear.

Estrogen receptor composed of two types, estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). Several studies have found that both forms of ERs are expressed throughout the brain and spinal cord, including the bed nucleus of the stria terminalis (BNTS), medial and cortical amygdaloid nuclei, preoptic area (POA), lateral habenula, periaqueductal gray (PAG), parabrachial nucleus, locus ceruleus, nucleus of the solitary tract, spinal trigeminal nucleus and superficial laminae of the spinal cord (Shughrue et al., 1997; Ogawa et al., 1998; Mitra et al., 2003; Shima et al., 2003). The immunohistochemical studies showed that the ERs contained cells in preoptic area and PAG also showed immunoreactivity for GABA (Herbison and Fenelon, 1995; Lovick and Paul, 1999). It was thus suggested that estrogen may affect the function of GABAergic system in these brain areas.

Estrogen and Anxiety

Nowadays, the information about the effects of estrogen on non-reproductive behavior such as cognitive performance, psychiatric disorders is widely published (Arpels, 1996; Sherwin, 1998; Walf and Frye, 2007; Ribeiro et al., 2009). Epidemiologic studies indicated that 8% to 47% of women undergoing the

menopausal transition experience anxiety symptom (Avis et al., 2001; Schmidt, 2005; Hickey et al., 2012), and estrogen replacement therapy in postmenopausal women can improve mood and feelings of general well being (Sherwin, 1998; Halbreich, 2003; Walf and Frye, 2007). These findings suggested the important role of estrogen in regulating anxiety disorder in women. However, the mechanisms of estrogen in controlling anxiety are not fully understood. To clarify the effect of estrogen on anxiety disorder, various models were developed to be used as an animal model. The ovariectomized mice or rats at different time point such as 7, 14 or 28 days were used (Bowman et al., 2002; Pandaranandaka et al., 2006; Walf and Frye 2007; Ribeiro et al., 2009). However, the inconsistent results have been found. For instance, Morgan and Pfaff (2001) using light dark transition test found that rats ovariectomized for 7 day and treated with estrogen were more anxious as they spent less time in the light compartment of the apparatus. On the other hand, the results of other studies showed that when the rats were ovariectomized for 21 or 28 days, the group that received estrogen was less anxious than the ovariectomized counter part (Andrade et al., 2005; Pandaranandaka et al., 2006). These inconsistent effects of estrogen may be influenced by the duration of the rat lacking of estrogen. However, there is no information regarding time of the estrogen withdrawal that can generate anxiety.

There were several studies demonstrating that estrogen could modulate enzyme or neurotransmitter function in the brain (Bethea et al., 2002; Nakamura et al., 2004; Pytel et al., 2007). Moreover, some studies mapped the distributions of estrogen receptor α and β in rat brain and showed that both subtypes were expressed in hippocampus, cortex, hypothalamus, midbrain and amygdala, these areas were related to mood regulation (Osterlund et al., 2000a, b). Therefore, estrogen may modulate anxiety by regulating enzyme or neurotransmitter functions in these brain areas.

It has been demonstrated that the sensitivity to benzodiazepine or GABA is changed across the estrous cycle. During estrus or diestrus phase, female mice were more responsive to the anxiolytic effects of diazepam with no effect during late diestrus, proestrus or metestrus phase (Carey et al., 1992; Reddy and Kulkarni, 1999). Similarly, decreases in the benzodiazepine sensitivity were observed in ovariectomized rats. After ovariectomy for 12 weeks, rats were more responsive to

diazepam than the group that was ovariectomized for 3 weeks (Picazo et al., 2006). This alteration was associated with increase anxiety in the shock-probe burying test (Picazo et al., 2006). These findings implied that estrogen may regulate anxiety by modulating the $GABA_A$ receptor in different brain areas related to anxiety.

Influence of estrogen on $GABA_A$ receptor subunit expression and $GABA_A$ receptor function

There is evidence indicating that steroid hormones are important regulators of $GABA_A$ receptor function. The actions of ovarian hormones on this receptor appear to occur as revealed by changes in the binding of $GABA_A$-benzodiazepine receptor ligands to membrane preparations or tissue sections from the brains of the rats in various phases of estrous cycle or ovariectomized rat (Herbison and Fenelon, 1995; Martin and Williams, 1995). In particular, the direct actions of progesterone metabolite may modulate $GABA_A$ receptor and its function (Gulinello et al., 2001; Gulinello and Smith, 2003; Amin et al., 2006; Smith et al., 2007). In addition, estrogen has been implicated in the regulation of ligand binding to the $GABA_A$ receptor (Schumacher et al., 1989; Herbison and Fenelon, 1995; Pierson et al., 2005; Jasnow et al., 2007; Xu et al., 2008). Although there is no evidence of this steroid as a direct allosteric modulator of $GABA_A$ receptor (Schumacher et al., 1989; Herbison and Fenelon, 1995; Pierson et al., 2005), the effects of estrogen on the $GABA_A$ receptor subunit expression and subsequent functional change of the inhibitory control systems have been found (Griffiths and Lovick, 2005; Lovick et al., 2005; Suda et al., 2008; Gouveia et al., 2009). The immunohistochemistry study by Lovick and colleagues (2005) showed that the α4, β1 and δ subunit mRNA of the $GABA_A$ receptor in female rats in late diestrus were higher than male or female in other phases of estrous cycle. Furthermore, the α4β1δ receptor expressed in the GABAergic interneuron in the PAG and the population of this receptor was increased during the late diestrus, when the levels of estrogen were low. These changes of $GABA_A$ receptor subunit expression associated with increased excitation of the neuronal circuit in the PAG (Lovick, 2008). In addition, *in vitro* and *in vivo* studies demonstrated that estrogen may regulate $GABA_A$ receptor subunit gene expression in different brain areas (Herbison and Fenelon, 1995; Pierson et al., 2005; Jasnow et al.,

2007; Xu et al., 2008; Noriega et al., 2010). Administration of estrogen for 7 days increased expression of α2, α3, β3 and ε but decreased expression of α5 subunit mRNA in teratocarcinoma cell (Pierson et al., 2005). The α2 and γ1 subunit mRNAs in the medial preoptic nucleus and bed nucleus of the stria terminalis were increased, whereas β3 subunit did not change after being treated with estrogen for 7 days in ovariectomized rats. The γ2 and δ mRNA in the hypothalamus were increased after estrogen administration (Follesa et al., 2002; Xu et al., 2008). Similarly, Noriega and co-workers (2010) found that estrogen can increase α1, α4, α5, β1 and γ2 $GABA_A$ receptor subunits mRNA expression in amygdala, hippocampus and hypothalamus of ovariectomized rhesus macaque. These results indicated that estrogen has specific effect on the pattern of $GABA_A$ receptor subunit mRNA expression and the alteration of $GABA_A$ receptor composition may alter $GABA_A$ receptor pharmacology (Diaz-Veliz et al., 2000; Gouveia et al., 2009). Thus, increased anxiety during low estrogen levels may be caused by the alteration of $GABA_A$ receptor subunit expression. However, the information about the effects of estrogen on the $GABA_A$ receptor gene expression and its relation to anxiety are not entirely clear.

E. Experimental models of anxiety

Animal model of anxiety can be divided into two main categories including conditioned and unconditioned responses (Steimer, 2011). For the conditioned response or conflict test such as vogel conflict test, Geller-Seifter conflict or fear-potentiated startle etc., the rats were exposed to punishment procedures that lead to suppress ongoing behavior (Safi et al., 2006). Vogel conflict test**,** the rats were water-deprived before test and during a test session, drinking was punished by a mild electric shocks delivered to the tongue (Millan and Brocco, 2003). In the Geller-Seifter test, during unpunished procedure, the rats were train to press a lever to obtain food. Then, the rats were submitted to punished-procedure by received an electric shock signaled by a light or auditory cue (Safi et al., 2006). For fear-potentiated startle, the rats were trained to expose to a neural stimulus such as light together with electric foot-shock. Then, after training session, the rats were exposure to intense sound. The startle response was potentiated by this unconditioned stimulus together with conditioned light stimulus (Bourin et al., 2007). In these conflict tests, the

response rate that occurs in the punished procedure was reduced, indicating the animal anticipating the punishment. Administration of anxiolytic drugs such as benzodiazepine agonist increased rates of punished without any change in the unpunished response (Millan and Brocco, 2003; Safi et al., 2006; Bourin et al., 2007).

For the unconditioned response, this test is involved the natural response to innate fear for example the elevated plus maze (EPM), the elevated T maze (ETM) and open field test, etc. (Litvin et al., 2008). The elevated plus maze test is based on a nature of the rats being fear of the open and high spaces, and used conflict between exploration and aversion to the open arms (Pellow et al., 1985). The measure of anxiety was the number of open arm entries and the time spent on the open arms. The anxiolytic drug increased the percentage of time spent on open arms relative to total time on the maze whereas the anxiogenic drug reverses this effect (Cole et al., 1995). The total number of entries was indicated a locomotor activity (Korte et al., 1999).

The pharmacological results indicated that the EPM is a mixed model in the sense that multiple defense reactions are displayed while the rat freely explores the apparatus. The elevated T-maze (ETM) is thus developed to discriminate conditioned and unconditioned fears, which have been related to generalize anxiety disorder and panic disorder, respectively. The pharmacological validation showed that acute injection with anxiolytic drug such as diazepam or ritanserin only impaired avoidance acquisition response (shortening latency to leave the enclosed arm) without changed on escape response (Graeff et al., 1998). This effect was associated with the clinical effectiveness of these drugs on the generalize anxiety disorder (Nutt, 1991). In addition, long-term treatment with 5-HT reuptake inhibitors such as imipramine, fluoxetine and chlorimipramine increased escape latency (Teixeira et al., 2000; Poltronieri et al., 2003) and these alterations also were in agreement with the clinical effectiveness of these drugs on the panic disorder (Johnson et al., 1995). These results indicated that the ETM model can assessed two different types of anxiety disorders i.e. generalize anxiety and panic disorders within the same animal.

For the open field test, the open field test is one of the popular model for investigate animal psychology (Litvin et al., 2008). The animal is exposed to a novel unknown environment by placed into the enclose apparatus. The following behavioral parameters were typically recorded for a period ranging from 2 to 20 min

(usually 5 min), to observe a number of behavior patterns including locomotion (number of crossings of the lines marked on the floor), frequency of rearing (sometimes termed vertical activity), grooming (protracted washing of the coat). Normally, rodents prefer the periphery of the apparatus more than the central parts of the open field. When the rodent stays in the periphery of the apparatus without enters into the center area called thigmotaxis and often interpreted as anxiety-like behavior. The increase of time spent in the central part or of the ratio central/total locomotion indicated of low anxiety-like behavior (Bourin et al., 2007). Many drugs such as anxiolytic drug, sedative, stimulant drugs have been investigated in the open field. An increase in central locomotion or in time spent in the central part of the open field without modification of total locomotion and of vertical exploration can be interpreted as an anxiolytic-like effect while drug that was decreased these parameter indicated anxiogenic effects. Drug that was increased locomotor activity can be considered a stimulant effect while decreased vertical and locomotor activities were related to sedation.

Altogether, it was likely that lack of estrogen for a period of time could induce anxiety-like behavior in the female; however, the length of estrogen deprivation affecting anxiety was not known. The mechanism of estrogen in regulating in anxiety could be occurred through various complicated mechanism; nevertheless, the GABAergic and serotonergic neural systems were likely to be of major interests. This was due to the fact that these two systems can be modulated by estrogen as demonstrated by pharmacological, behavioral and biochemical studies. Moreover, in clinical cases, the drugs affecting GABA and serotonin levels such as benzodiazepine agonist and SSRIs were widely used. Therefore, in the current study, the effect of estrogen in modulating anxiety was focused on GABA and serotonergic activity. The anxiety levels were measured with ETM, as it was claimed that this model was able to discriminate different types of anxiety i.e. generalize anxiety disorder and panic disorder. In addition, the open field test was also utilized for the measurement of locomotor activity which could not be measured with the ETM, to warrant that the anxiety seen in the ETM was not due to the movement failure of the animal. The

results from this experiment will provide more insight knowledge on the mechanisms of estrogen on regulating anxiety behavior.

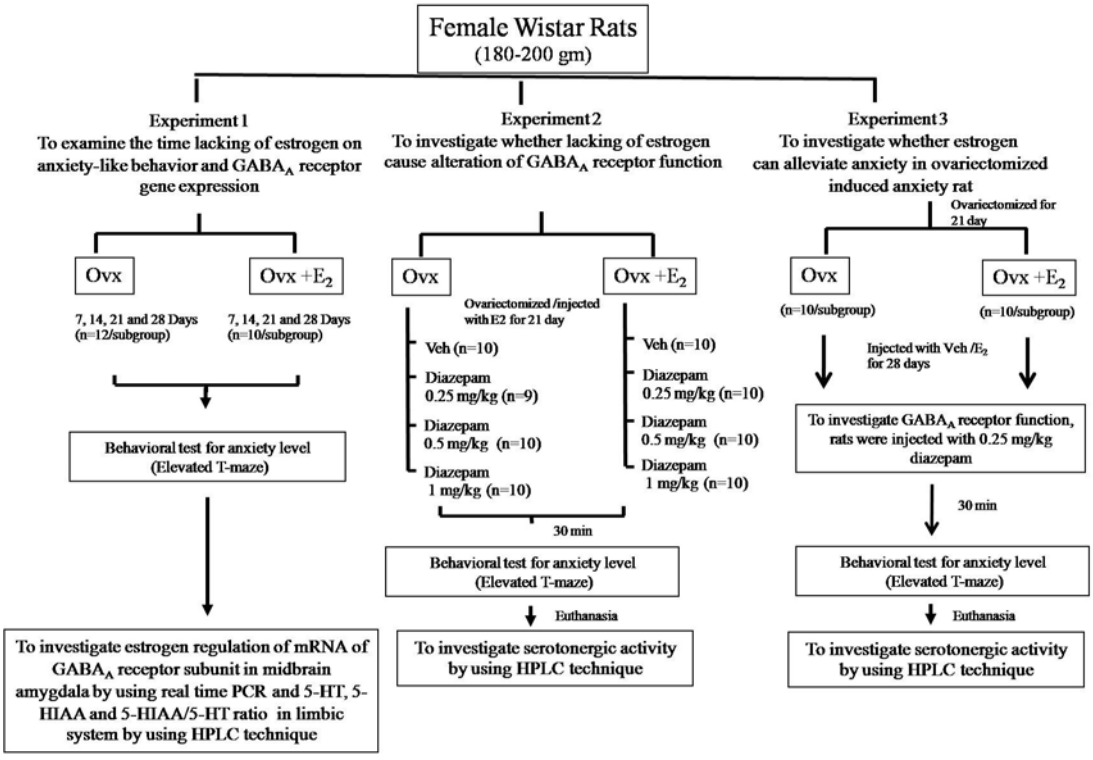
CHAPTER III
MATERIALS AND METHODS

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.

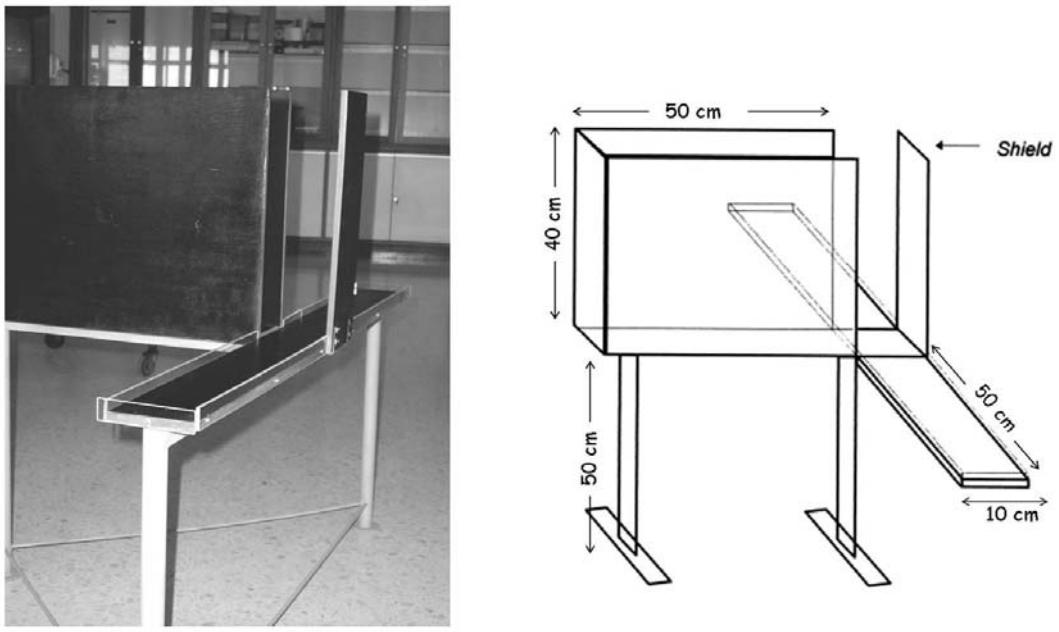


Figure 3-1 The elevated T-maze composed of two open- and one closed-arms of equal dimension (10 x 50 cm), connected by the center platform (10 x 10 cm). The closed-arm was enclosed by a 50-cm wall, and the maze was elevated 50 cm above the floor.

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.

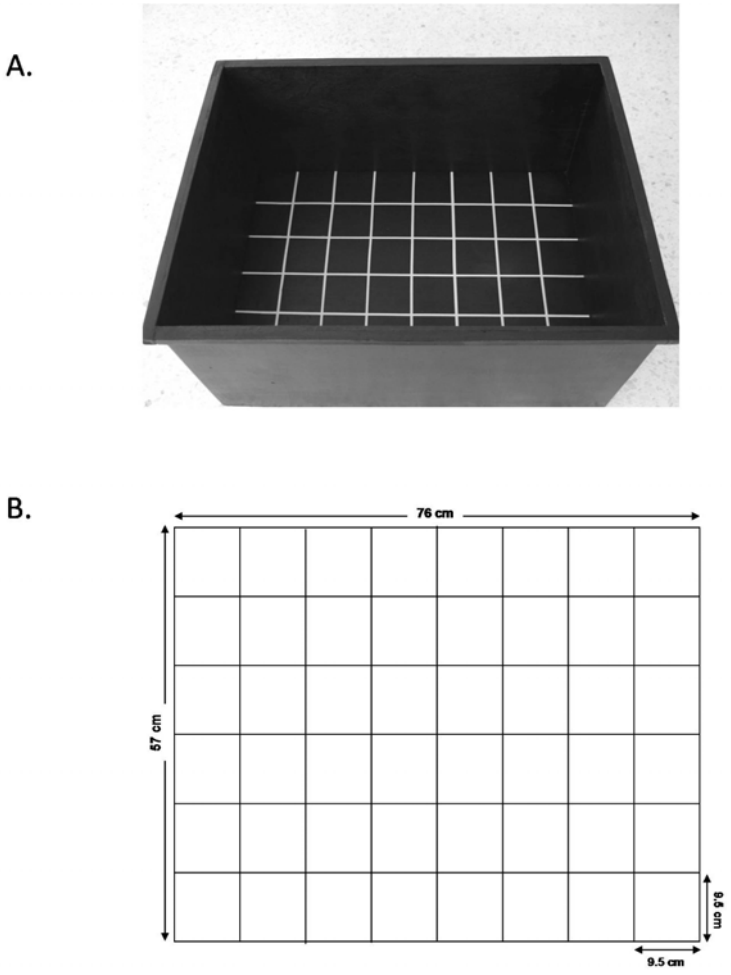


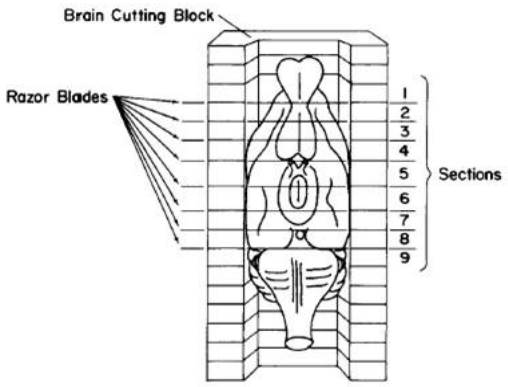
Figure 3-2 The open field (A) was a rectangular box of dimension (57 x 76 x 50 cm), in which the floor (B) was divided into 6 x 8 squares (9.5 x 9.5 cm).

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

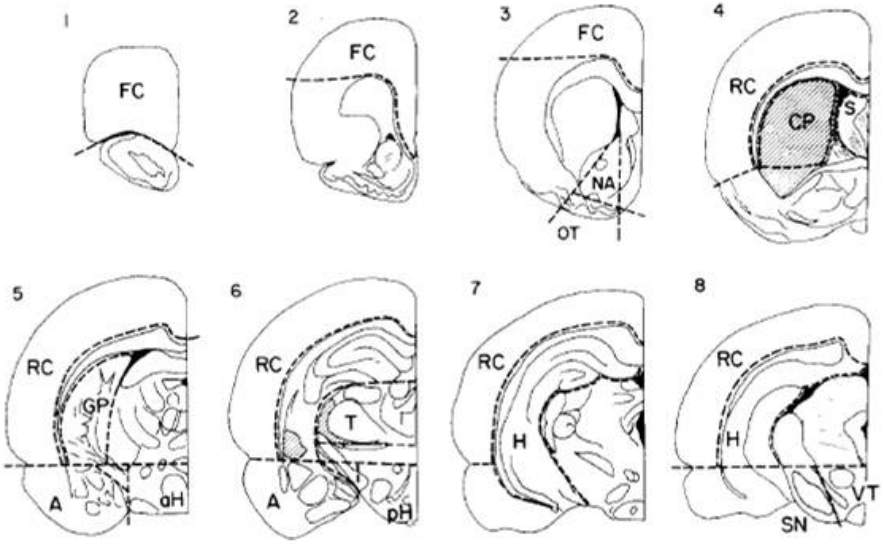


Figure 3-3 **(A)** Diagrammatic representation of brain cutting block illustrating orientation of brain and placement of razor blades to obtain coronal brain sections. The numbers on the right refer to brain sections. **(B)** Diagrammatic representation of coronal brain sections from which brain regions 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.

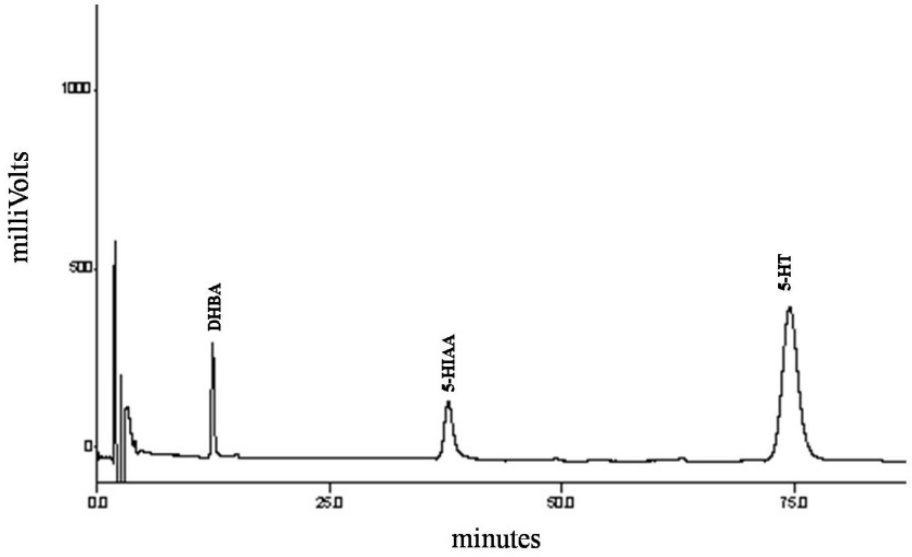


Figure 3-4 The chromatogram represents peaks of standard DHBA, 5-HIAA and 5-HT measured by HPLC-EC. The retention times of DHBA, 5-HIAA and 5-HT were approximately 12.38, 37.75 and 74.41 minutes, respectively.

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

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

In this part, the rats were divided into 2 groups, Ovx and E_2 groups and randomly selected at day 7, 14, 21 and 28 after ovariectomy to determine anxiety levels by using ETM. Body weight and food intake were measured daily. Uterine weight in all groups of animals was weighed immediately after sacrifice.

1.1 The effects of time of estrogen deprivation on body weight, food intake, and uterine weight

The results of physiological change after ovariectomy were summarized in table 4-1. In the beginning of the experiment, the body weight did not differ among groups; however, after ovariectomy, the body weight and the percent change of the body weight of the Ovx group were higher than the E_2 group at all time points ($P < 0.05$). The daily food intake was not significant difference between groups.

The lacking of ovarian hormones was confirmed by the reduction in the uterine weight (UW) and the ratio of uterine weight to body weight (%UW/BW) in the Ovx rats. The UW and %UW/BW of Ovx groups were lower than E_2 groups at all time points ($P < 0.05$) (Table 4-2). Moreover, the reductions in the UW and %UW/BW of Ovx groups were decreased in a time-dependent manner (UW: [$F(3, 47) = 27.66$, $P < 0.0001$]; %UW/BW: [$F(3, 47) = 54.45$, $P < 0.0001$]) (Table 4-2), demonstrating the negative correlations between UW or %UW/BW with the number of day following ovariectomy [UW: $r^2 = 0.7542$; $P < 0.0001$, $N = 48$; %UW/BW: $r^2 = 0.8585$; $P < 0.0001$, $N = 48$].

Table 4-1 The body weights, the percent change of body weight and the daily food intake in Ovx and E_2 rats at day 7-, 14-, 21- and 28- post-ovariectomy.

Parameters	7 Day	14 Day	21 Day	28 Day
Beginning weight (gm)				
Ovx	201.46±1.29	196.46±2.31	200.42±2.28	201.46±1.81
E_2	205.21±1.83	197.50±2.42	199.55±2.69	204.77±2.75
End weight(gm)				
Ovx	220.00±1.95	236.46±3.53	252.50+4.23	280.42±5.41
E_2	213.75±1.86***	216.88±3.80***	223.41±2.67***	247.50±5.27***
Percent change of body weight				
Ovx	9.19±0.53	20.45±1.76	26.19±2.64	39.19±2.43
E_2	4.18±0.49***	9.82±1.42**	11.87±0.71*	20.84±1.82***
Daily food intake (gm/d)				
Ovx	12.30±0.32	12.22±0.74	12.48±0.59	12.68±0.76
E_2	10.55±0.92	11.16±0.75	12.47±2.58	12.94±0.68

Data presented as mean ± S.E.M., *P<0.05, **P<0.005 and ***P<0.0001, significantly different from corresponding Ovx groups at the same time point using Student's unpaired *t*-test. n = 10-12 to each subgroup.

Table 4-2 The uterine weight, percentage of uterine weight to body weight ratio in 7-, 14-, 21- and 28-days Ovx rats.

Parameters	7 Day	14 Day	21 Day	28 Day
UW (gm)				
Ovx	0.147± 0.004 [a]	0.128± 0.003 [b]	0.115 ±0.002 [c]	0.112± 0.003 [c]
E_2	0.311± 0.012***	0.340± 0.013***	0.326± 0.008***	0.420± 0.010***
UW / BW (%)				
Ovx	0.067±0.002 [a]	0.054±0.001[b]	0.045±0.001[c]	0.042±0.001[c]
E_2	0.146±0.007***	0.156±0.007***	0.145±0.004***	0.170±0.006***

Data presented as mean ± S.E.M., ***P<0.0001, significantly different from corresponding Ovx groups at the same time point using Student's unpaired *t*-test.
[a,b,c] Different letters denoted significant difference between time points within treatment at P<0.05, ANOVA followed by Duncan's multiple comparison test. n = 10-12 to each subgroup.

1.2. The effects of duration of estrogen deprivation on anxiety-like behavior and locomotor activity

The anxiety levels of the Ovx rats as measured by the ETM are shown in figure 4-1. The inhibitory avoidance trials from the ETM tests showed a significant effect of day after ovariectomy [F (3, 143) = 13.73, P < 0.0001] and trials [F (2, 141) = 17.11, P < 0.0001]. The Duncan post hoc test revealed that the baseline latency was not different among days. Significant differences were seen in the avoidance latencies trial 1 and 2, the rats that were ovariectomized for 21- and 28-days took more time to leave the closed arm than those ovariectomized for 7-and 14- day [F (3, 46) = 9.72, P < 0.001] (Figure 4-1A). In addition, the positive correlation between the avoidance latency in trial 2 and the number of day following ovariectomy was found [r^2 = 0.62259; P < 0.0001, N= 48]. For the E_2 groups, the number of day following ovariectomy had no significant effect on anxiety behavior in the ETM [F (3, 45) = 1.19, P = 0.3238] (Figure 4-1B).

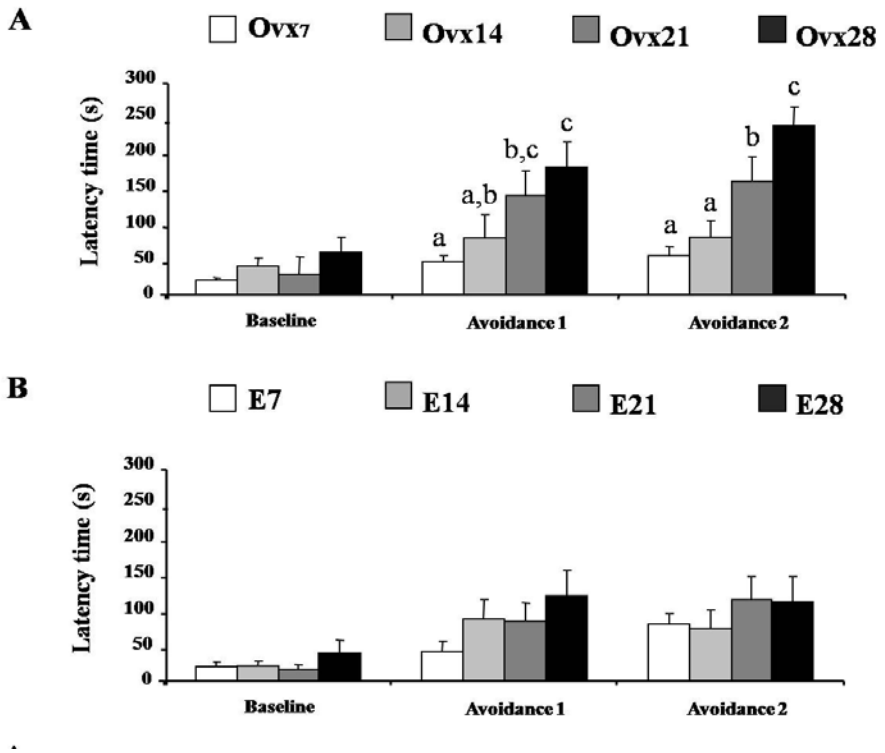


Figure 4-1 The effects of estrogen deprivation on anxiety-like behavior at each time point in (A) Ovx groups and (B) E_2 groups. Data present as mean $\pm$ SEM; ^{a,b,c} Different letters indicate statistical differences ($P<0.05$) among groups in the same trial. n = 10-12 for each subgroup.

For the escape test, there was no significant difference among subgroups ($P > 0.05$) (Figure 4-2A). The locomotor activity, the total number of line crossed in the open field during 5 min was not differed among groups ($P > 0.05$; Figure 4-2B), indicating that the behaviors seen in the ETM were not affected by treatments.

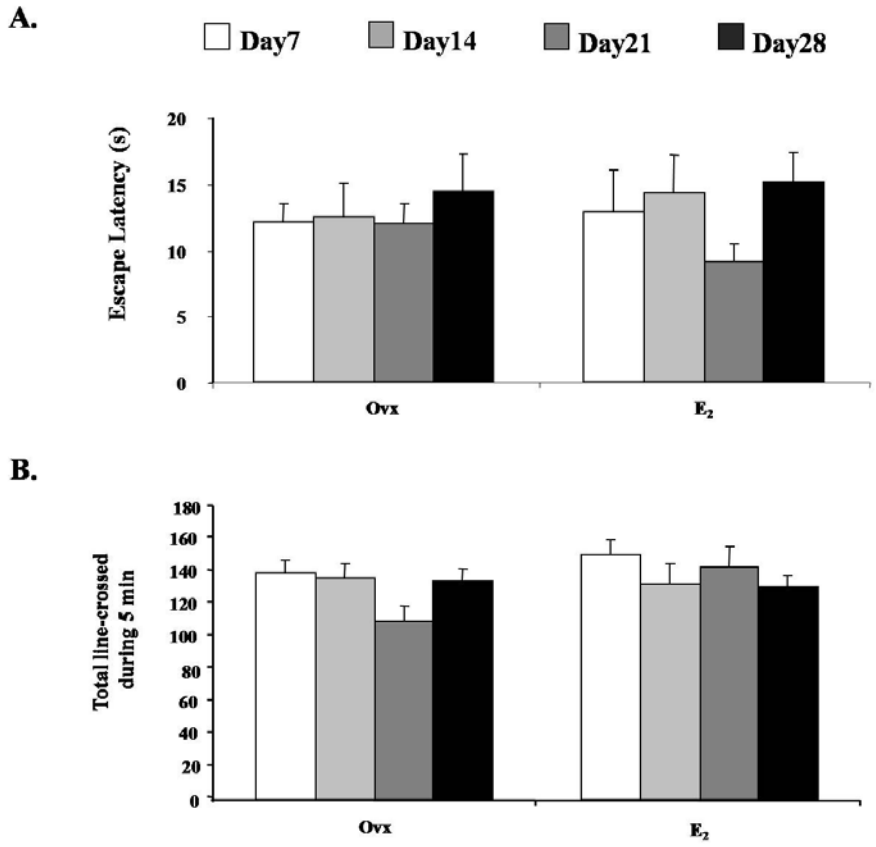


Figure 4-2 The effects of time of estrogen deprivation on (A) escape latency in ETM and (B) locomotor activity in the open field**.** Data present as mean $\pm$ SEM. n = 10-12 for each subgroup.

1.3. The effects of time of estrogen deprivation on serotonergic activity in brain associated with anxiety

After behavioral test, the rat's brains were rapidly removed for measurement of 5-HT and 5-HIAA levels by HPLC technique. Figure 4-3 and 4-4 represent the example of chromatogram of 5-HT and 5-HIAA in midbrain of Ovx and E_2 rats at different time after ovariectomy.

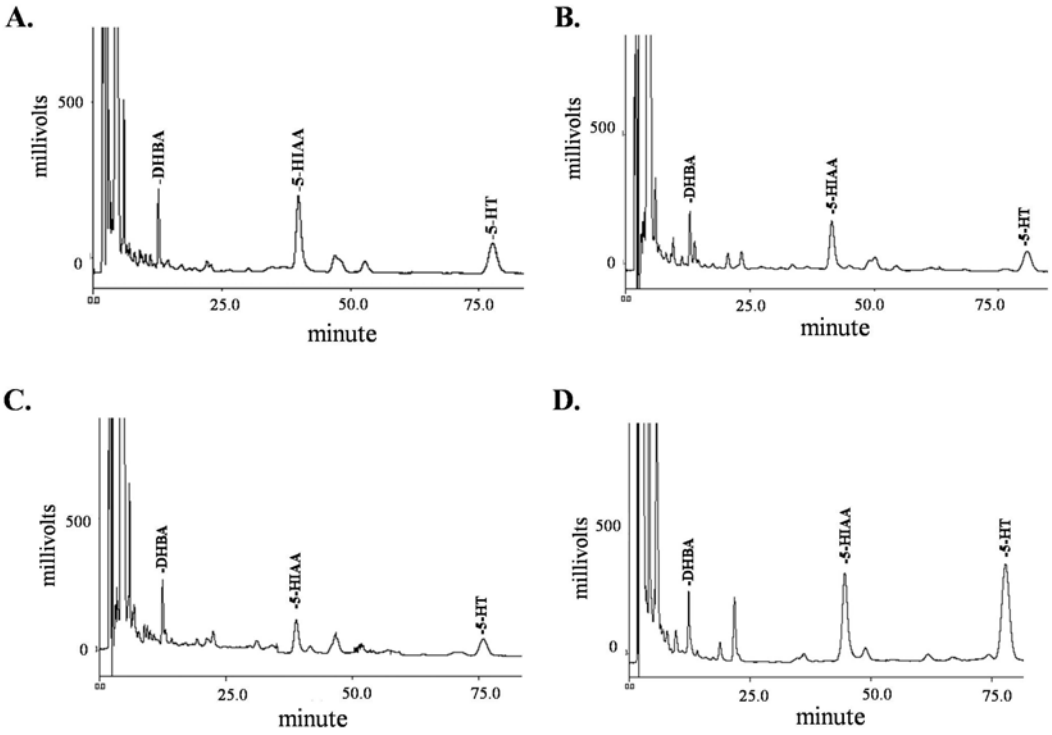


Figure 4-3 The chromatograms represent 5-HT and 5-HIAA levels in midbrain of (A) Ovx7, (B) Ovx14, (C) Ovx21 and (D) Ovx28 groups measured by HPLC-EC. The retention times of 5-HIAA and 5-HT were approximately 37.75 and 75.41, respectively.

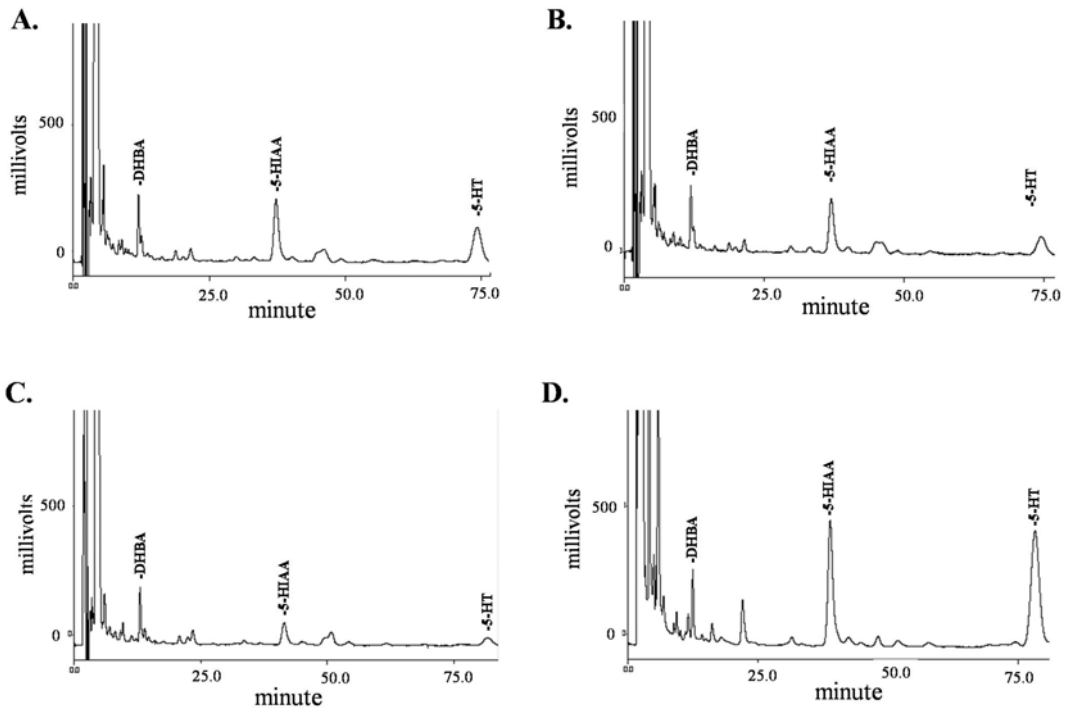


Figure 4-4 The chromatograms represent 5-HT and 5-HIAA levels in midbrain of (A) E7, (B) E14, (C) E21 and (D) E28 groups measured by HPLC-EC. The retention times of 5-HIAA and 5-HT were approximately 37.75 and 75.41, respectively.

In the midbrain, two-way ANOVA revealed a significant effect of treatment and day for 5-HT and 5-HIAA levels (5-HT: treatment [F (1, 46) = 9.02, P = 0.0046]; day [F (3, 44) = 12.05, P < 0.0001]; 5-HIAA: treatment [F (1, 46) = 9.59, P = 0.0036]; day [F (3, 44) = 7.15, P = 0.0006]). The levels of 5-HT and 5-HIAA in the Ovx groups were significantly higher than the E_2 groups (Figure 4-5A, B). There was no significant effect of treatment or day on 5-HIAA/5-HT ratio (Figure 4-5C). When the comparison was made between days within the same group, the 5-HT and 5-HIAA levels at day 21 after ovariectomy in the Ovx group were lowest with significant difference from those at days 14 and 28 for 5-HT and at day 28 for 5-HIAA (Figure 4-5A, B). The 5-HIAA/5-HT ratio at day 21 in the Ovx group was thus highest with significant difference from those at day 28 (Figure 4-5C). In the E2 groups, the levels of 5-HT and its metabolite were significantly increased at day 28 when compared to other days (Figure 4-5A, B) with no difference in the ratio of 5-HIAA/5-HT.

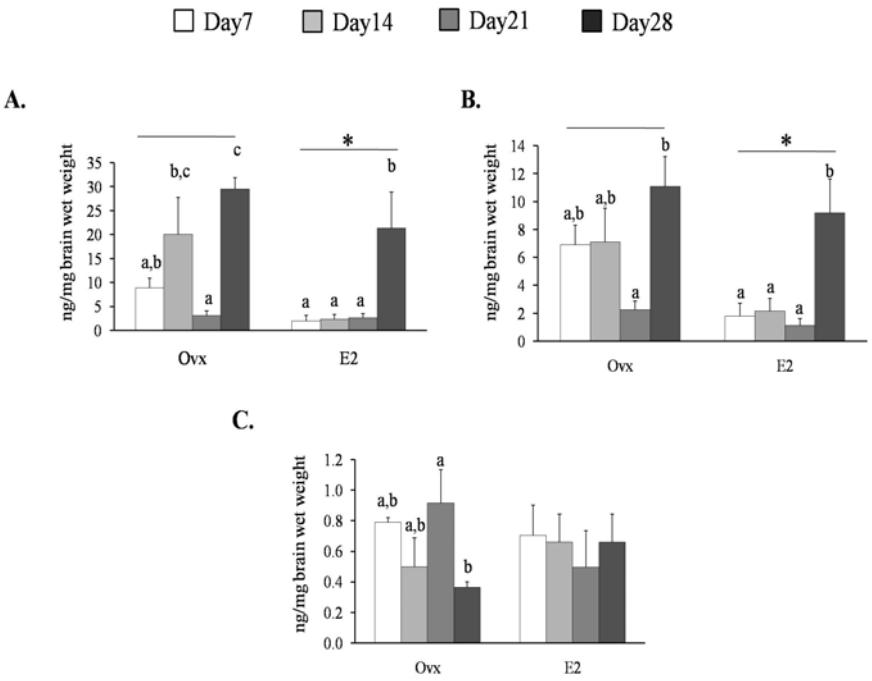


Figure 4-5 The effect of time of estrogen deprivation on (A) 5-HT, (B) 5-HIAA levels and (C) 5-HIAA/5-HT ratios in midbrain. Data presented as mean ± SEM; *P< 0.05 significant difference from Ovx groups, two-way ANOVA. ^{a,b,c} Different letters denoted significant different at P< 0.05, one-way ANOVA followed by Duncan's multiple comparison test. n = 6 for each subgroup.

In the amygdala, two-way ANOVA showed a significant effect of day for 5-HT [F (3, 44) = 44.85, P < 0.0001], 5-HIAA [F (3, 44) = 13.47, P < 0.0001] and 5-HIAA/5-HT ratio [F (3, 44) = 6.71, P = 0.0010] (Figure 4-6). Moreover, there was interaction between treatment and day on the ratio of 5-HIAA/5-HT [F (3, 44) = 3.68, P = 0.0203]. When the comparison was made between days within the same group, the levels of 5-HT and 5-HIAA levels in the rat that were ovariectomized for 28 days were significantly higher than those ovariectomized for 7, 14 and 21 days (Figure 4-6A,B). The 5-HIAA/5-HT ratio in Ovx group was significantly decreased in time dependent manner (Figure 4-6C). In E_2 groups, the 5-HT and 5-HIAA levels were significantly increased at day 28 when compared to other days (Figure 4-6A, B) with no difference in the ratio of 5-HIAA/5-HT (Figure 4-6C).

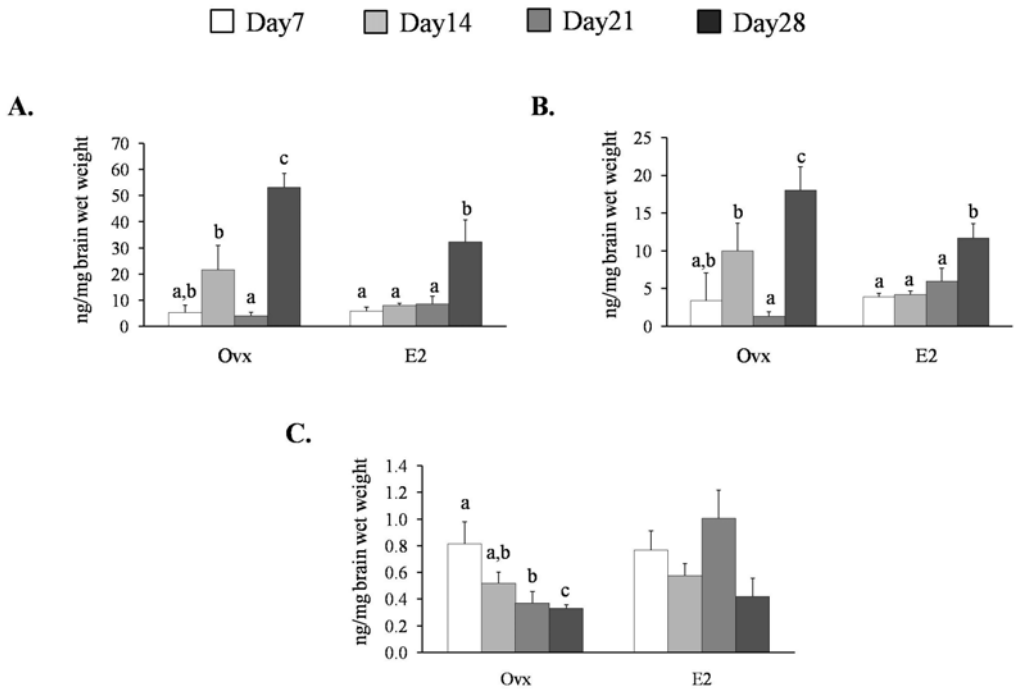


Figure 4-6 The effect of time of estrogen deprivation on (A) 5-HT, (B) 5-HIAA levels and (C) 5-HIAA/5-HT ratios in amygdala. Data presented as mean ± SEM; *P< 0.05 significant difference from Ovx groups, two-way ANOVA. [a,b,c] Different letters denoted significant different at P< 0.05, one-way ANOVA followed by Duncan's multiple comparison test. n = 6 for each subgroup.

In the frontal cortex, two-way ANOVA showed a significant effect of day for 5-HT and 5-HIAA levels (5-HT: [F (3, 44) = 33.44, P < 0.0001]; 5-HIAA: [F (3, 44) = 8.58, P = 0.0002] (Figure 4-7). When the comparison was made between days within the same group, the 5-HT and 5-HIAA levels at day 28 after ovariectomy in the Ovx group were significantly increased when compared to other days with no difference in the ratio of 5-HIAA/5-HT (Figure 4-7A, B and C). In the E_2 groups, the levels of 5-HT and 5-HIAA were increased at day 28 compare to days 7, 14 and 21 but significantly only for the 5-HT levels (Figure 4-7A, B). Consequently, the ratio of 5-HIAA/5-HT was not significant different in these groups (Figure 4-7C).

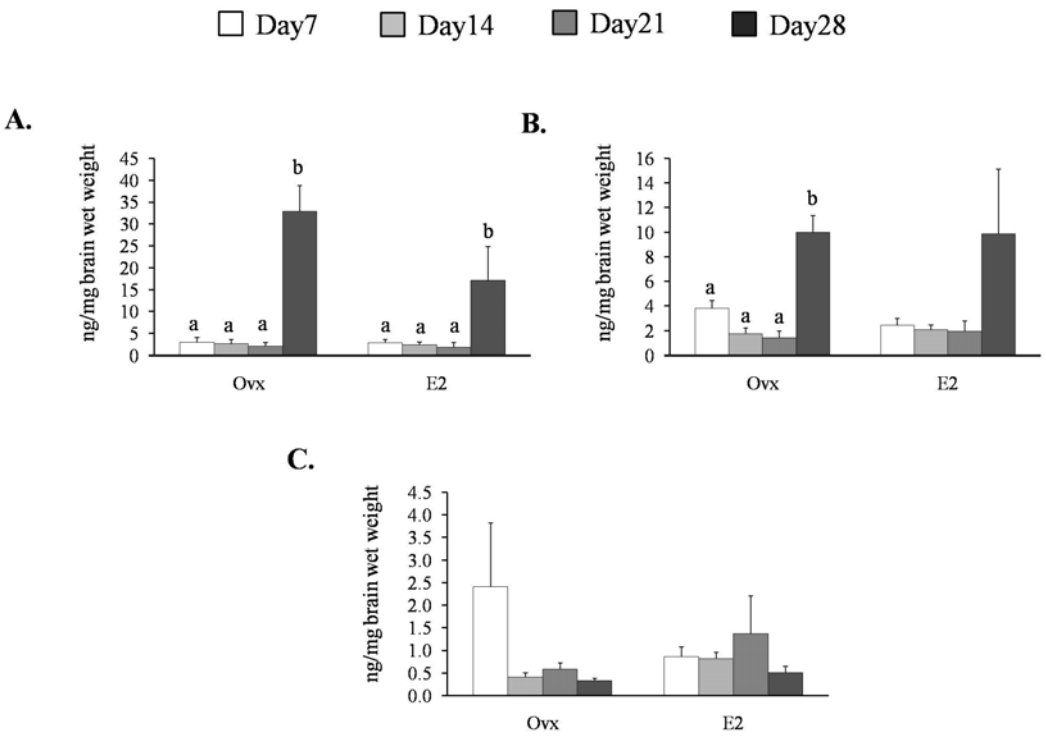


Figure 4-7 The effect of time of estrogen deprivation on (A) 5-HT, (B) 5-HIAA levels and (C) 5-HIAA/5-HT ratios in frontal cortex. Data presented as mean ± SEM; [a,b,c] Different letters denoted significant different at P< 0.05, one-way ANOVA followed by Duncan's multiple comparison test. n = 6 for each subgroup.

In the hippocampus, two-way ANOVA revealed a significant effect of day for 5-HT and 5-HIAA levels (5-HT: [F (3, 44) = 24.84, P < 0.0001]; 5-HIAA levels [F (3, 44) = 12.69, P < 0.0001]) (Figure 4-8A, B) with no significant effect on the 5-HIAA/5-HT ratio (Figure 4-8C). When the comparison was made between days within the same group, the 5-HT and 5-HIAA levels at day 28 after ovariectomy in the Ovx groups were significantly higher than those at day 7, 14 and 21 (Figure 4-8A, B) with no significant difference in the ratio of 5-HIAA/5-HT (Figure 4-8C). For the E_2 groups, the 5-HT and 5-HIAA were highest at day 28; but the significant was found between day 28 to day 7 and 21 for 5-HT and between day 28 to day 7 for 5-HIAA (Figure 4-8A, B). In this group, there was no difference in the ratio of 5-HIAA/5-HT (Figure 4-8C).

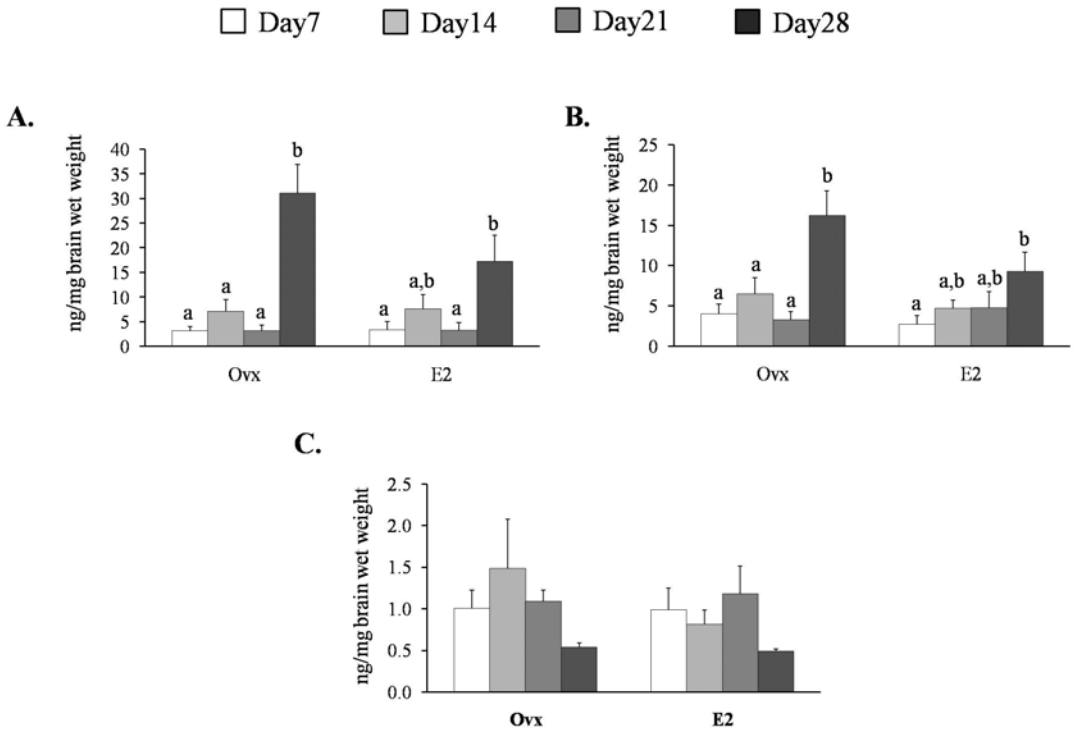


Figure 4-8 The effect of time of estrogen deprivation on (A) 5-HT, (B) 5-HIAA levels and (C) 5-HIAA/5-HT ratios in hippocampus. Data presented as mean ± SEM; ^{a,b,c} Different letters denoted significant different at P< 0.05, one-way ANOVA followed by Duncan's multiple comparison test. n = 6 for each subgroup.

In the nucleus accumben, the levels of 5-HT, 5-HIAA and ratio of 5-HIAA/5-HT are shown in figure 4-9. The two-way ANOVA revealed a significant effect of day for 5-HT and 5-HIAA levels (5-HT: [F (3, 44) = 4.45, P = 0.0086]; 5-HIAA levels [F (3, 44) = 4.34, P = 0.0097]). The 5-HT and 5-HIAA levels at day 28 in both groups were higher than at days 7, 14 and 21 with no effect on 5-HIAA/5-HT ratio. When the comparison was made between days within the same group, the significant difference was found only in the Ovx group in that the 5-HIAA/5-HT ratio at day 14 was higher than those at days 7, 14 and 28 (Figure 4-9C).

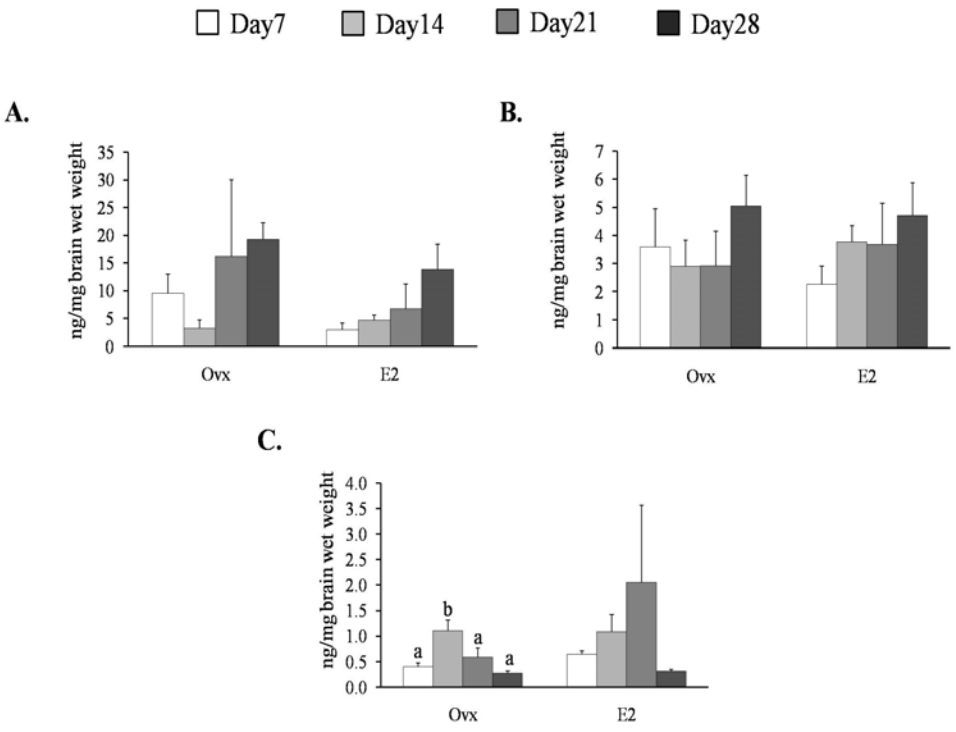


Figure 4-9 The effect of time of estrogen deprivation on (A) 5-HT, (B) 5-HIAA levels and (C) 5-HIAA/5-HT ratios in nucleus accumbens. Data presented as mean $\pm$ SEM; ^{a,b,c} Different letters denoted significant different at $P < 0.05$, ANOVA followed by Duncan's multiple comparison test. n = 6 for each subgroup.

In the septum, the levels of 5-HT, 5-HIAA and ratio of 5-HIAA/5-HT are shown in figure 4-10. There was no significant effect of treatment or day for 5-HT, 5-HIAA and 5-HIAA/5-HT ratio. When the comparison was made between days within the same group, the levels of 5-HT and 5-HIAA and the ratio of 5-HIAA/5-HT in the Ovx group were not different between days. In the E_2 groups, the levels of 5-HT and 5-HIAA were not different between days. However, the 5-HIAA/5-HT ratio at day 14 was lower than those at day 7 and 21 (Figure 4-10C).

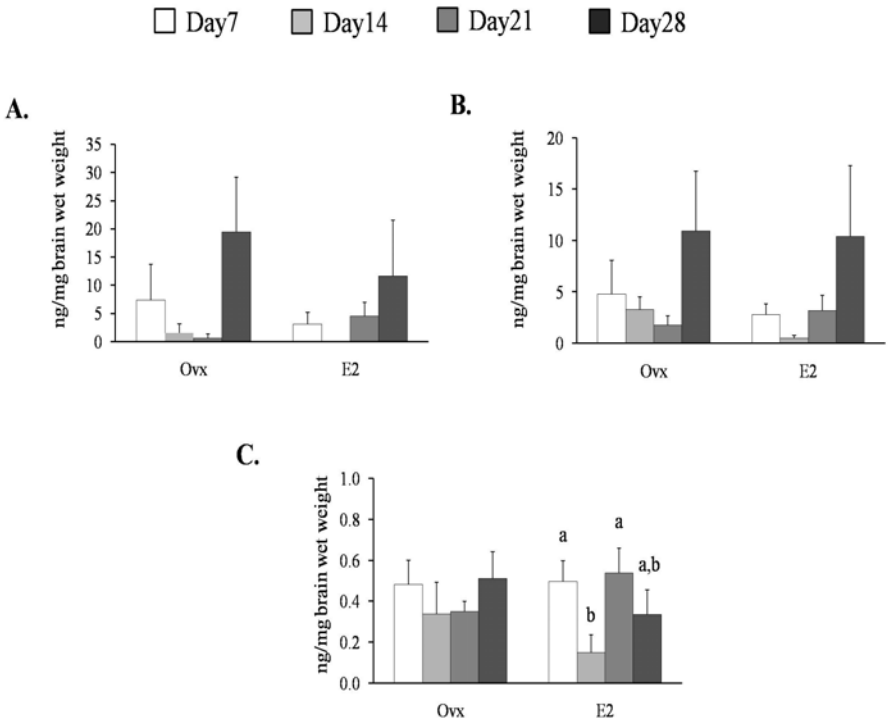


Figure 4-10 The effect of time of estrogen deprivation on (A) 5-HT, (B) 5-HIAA levels and (C) 5-HIAA/5-HT ratios in septum. Data presented as mean $\pm$ SEM; ^{a,b,c} Different letters denoted significant different at $P < 0.05$, ANOVA followed by Duncan's multiple comparison test. n = 6 for each subgroup.

In the anterior hypothalamus, the levels of 5-HT, 5-HIAA and ratio of 5-HIAA/5-HT are shown in figure 4-11. The statistical analyses revealed no significant differences in the levels of 5-HT, 5-HIAA or the ratio of 5-HIAA/5-HT within the Ovx and E_2 groups.

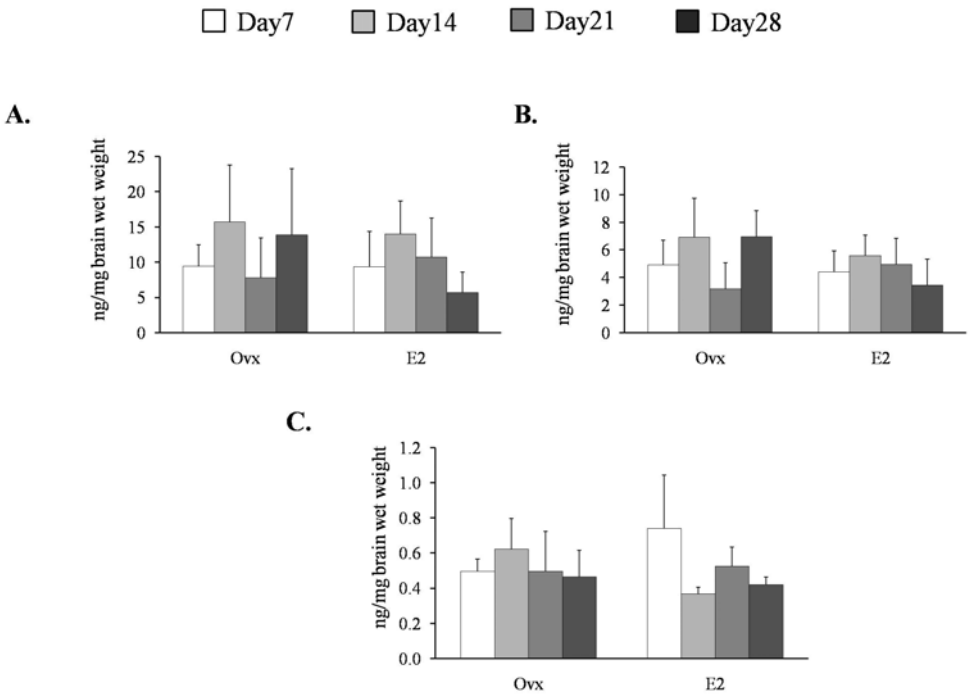


Figure 4-11 The effect of time of estrogen deprivation on (A) 5-HT, (B) 5-HIAA levels and (C) 5-HIAA/5-HT ratios in anterior hypothalamus. Data presented as mean $\pm$ SEM; n = 6 for each subgroup.

1.4. The effects of time of estrogen deprivation on $GABA_A$ receptor subunits gene expression in brain associated with anxiety-like behaviors

In this part, the Ovx or E_2 rats were randomly selected at day 7, 14, 21 and 28 after ovariectomy to investigate the $GABA_A$ receptor subunit gene expression in the midbrain and amygdala. The specificity of each primer used in this study was confirmed by performing a high resolution gel electrophoresis and a dissociation curve at the end of PCR. As shown in figure 4-12 and 4-13, a single band and a single peak were evidenced in an agarose gel electrophoresis and dissociation curves, respectively; indicating primers specificity.

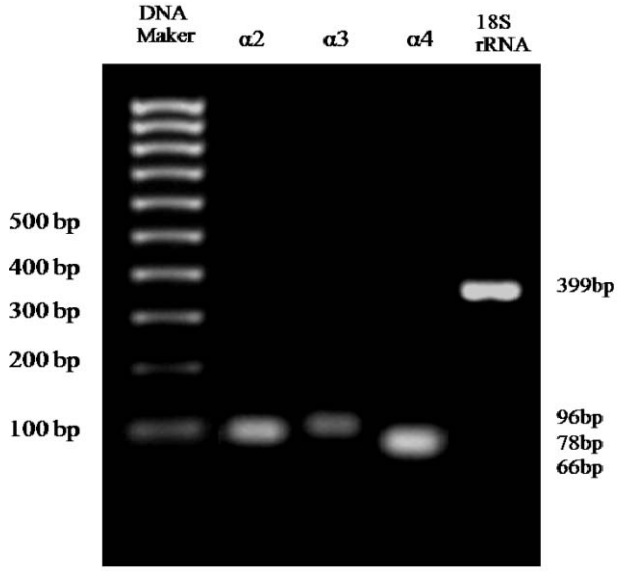


Figure 4-12 Agarose gel electrophoresis of PCR amplification products of $GABA_A$ receptor α2, α3, α4 subunits and 18s rRNA. The numbers on the left indicate the DNA ladder; while, the numbers on the right indicate the size(s) of each PCR product.

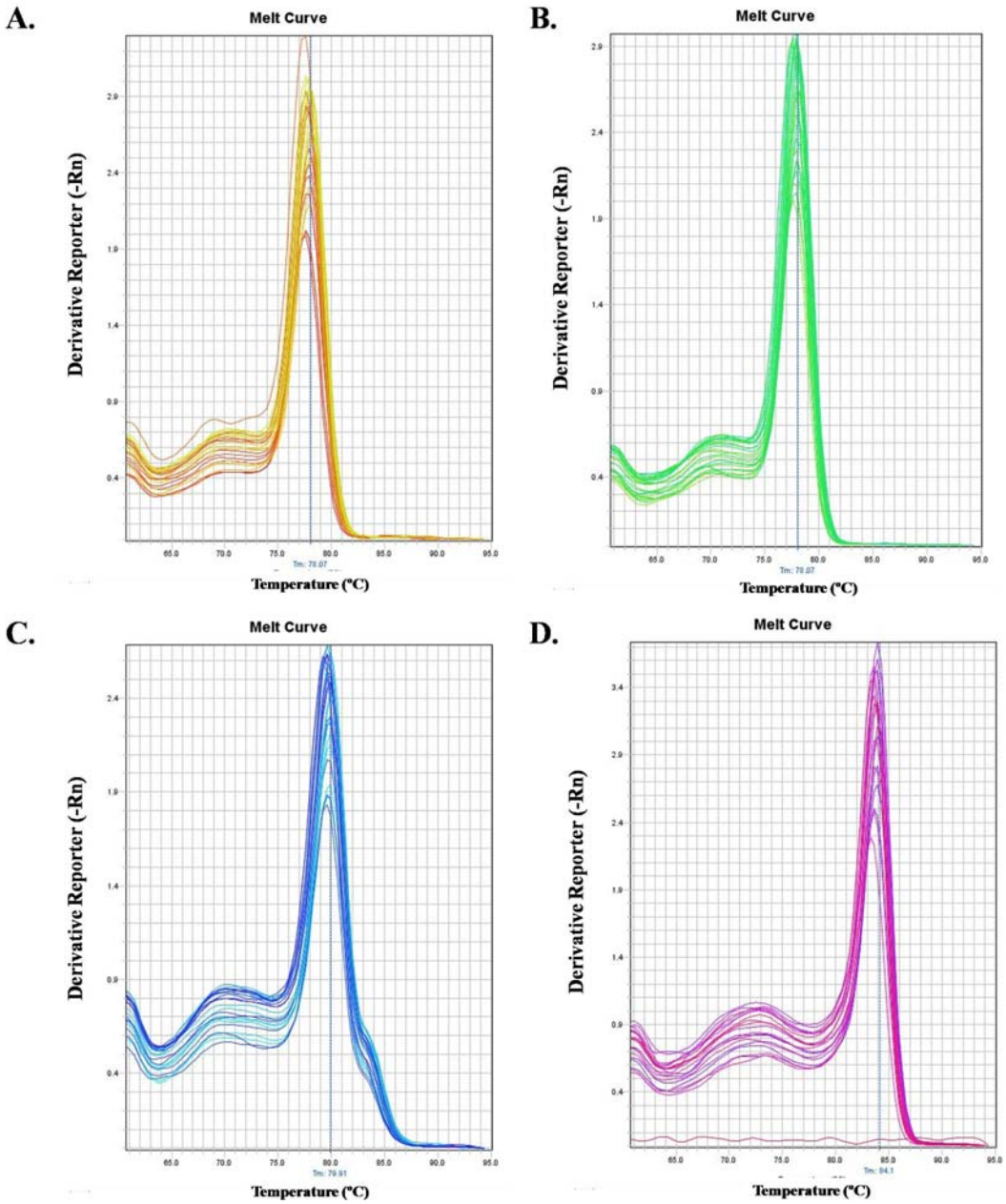


Figure 4-13 Dissociation curves of PCR amplification products of $GABA_A$ receptor (A) α2, (B) α3, (C) α4 subunits and (D) 18s rRNA.

Relative expressions of $GABA_A$ α2, α3 and α4 subunit mRNA in the midbrain are shown in figure 4-14. The two-way ANOVA revealed that the expression of $GABA_A$ α2 and α3 subunit mRNA in the Ovx groups were higher than the E_2 groups [α2: $F(1, 22) = 4.81$, $P = 0.0435$; α3: $F(1, 32) = 5.0$, $P = 0.0341$] (Figure 4-14A, B); while there was no significant effect of treatment or day on $GABA_A$ α4 subunit mRNA expression (Figure 4-14C). When the comparison was made between days within the same group, the expression levels of α2, α3 and α4 subunit mRNA in the E_2 groups were relatively stable and were not significant difference between days. However, in the Ovx groups, the expressions were fluctuated depending on day post-ovariectomy. For the α2 subunit mRNA, the relative expression was increased at day 28 but not significant different; and for the α3 and α4 subunit mRNA, similar pattern was found with the increased expression at day 21 compared to other days but significant effect was found only for α4 subunit mRNA (Figure 4-14C).

Relative expression of $GABA_A$ α2, α3 and α4 subunit mRNA in the amygdala are shown in figure 4-15. The two-way ANOVA revealed no significant effect of treatment or day after ovariectomy for $GABA_A$ α2, α3 and α4 subunit mRNA (Figure 4-15). Interestingly, the patterns of expressions were similar between subunits of each treatment; in the E_2 groups, the expression was relatively higher at day 7 then decreased and gradually increased later. In the Ovx groups, the patterns were differed from those of E_2, it was increased from day 7 to 14 and then abruptly decreased at day 21 and likely to be continually decreased at day 28.

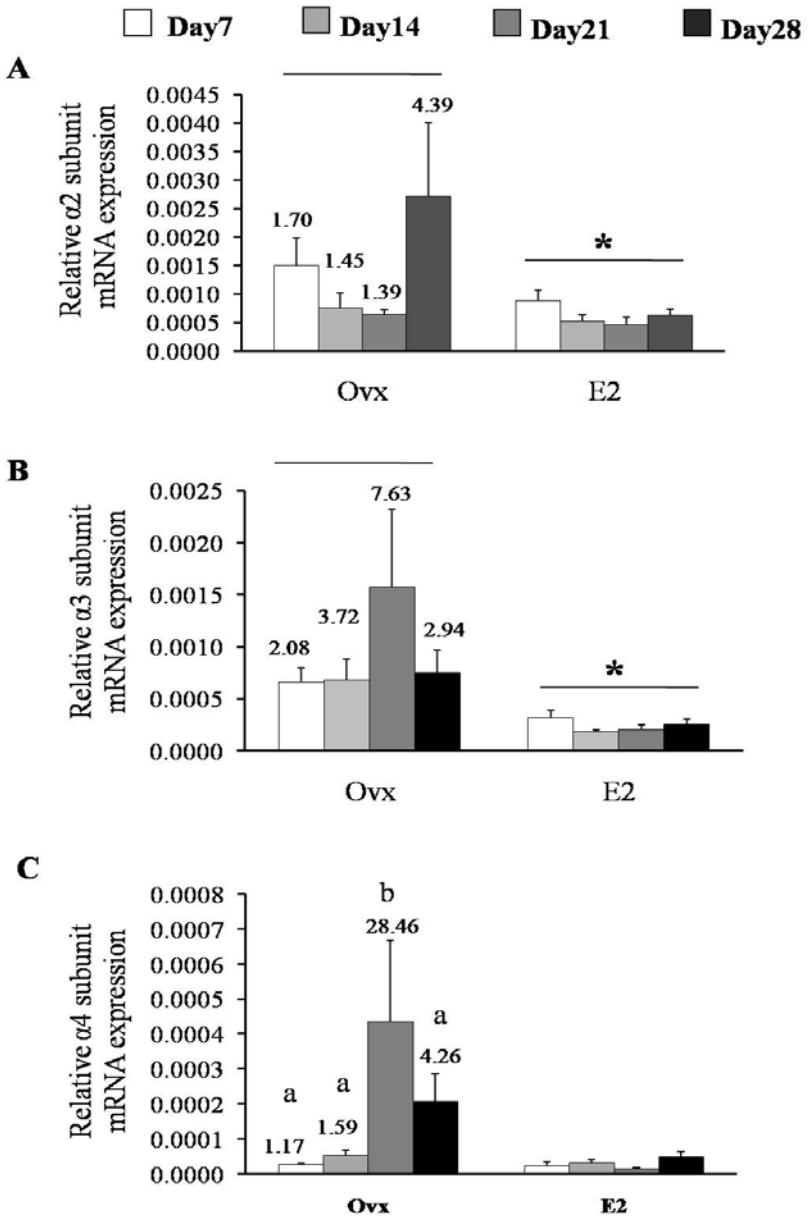


Figure 4-14 The effect of time of estrogen deprivation on $GABA_A$ receptor (A) α2-, (B) α3- and (C) α4- subunit gene expressions in midbrain. Data presented as mean ± SEM; * significant difference ($P < 0.05$) from Ovx groups, two-way ANOVA. [a,b] Different letters denoted significant different within group at $P < 0.05$, ANOVA followed by Duncan's multiple comparison test. The number above each bar represents fold change from E_2 at the same time point. n = 3-6 for each subgroup.

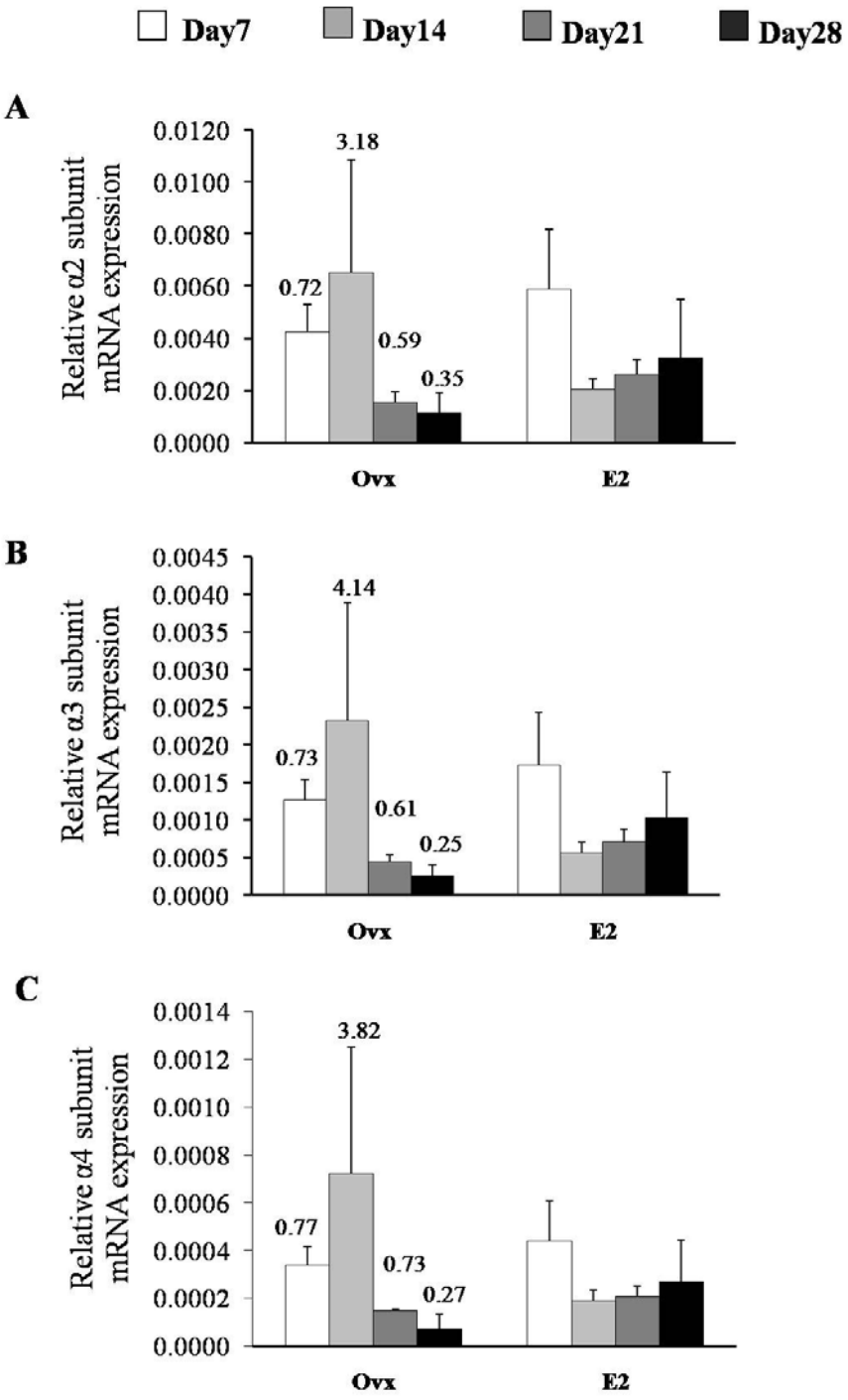


Figure 4-15 The effect of time of estrogen deprivation on $GABA_A$ receptor (A) α2-, (B) α3- and (C) α4- subunit gene expressions in amygdala. Data presented as mean ± SEM; the number above each bar represents fold change from E_2 at the same time point. n = 3 for each subgroup.

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

In this part, after ovariectomy for 21 days, both Ovx and E_2 groups were administered with saline or various dosages of benzodiazepine agonist (diazepam; 0.25, 0.5 or 1 mg/kg BW) 30 minutes before behavioral tests.

2.1 The effect of benzodiazepine agonist on the anxiety-like behaviors and locomotor activity

The effect of benzodiazepine agonist on anxiety-like behavior in the Ovx rats as measured by the ETM are shown in figure 4-16A. The inhibitory avoidance trials from the ETM tests revealed a significant effect of dose [$F(3, 116) = 4.71$, $P = 0.0040$] and trials [$F(2, 117) = 20.36$, $P < 0.0001$]. When the comparison was made between doses of diazepam within the same trial, the baseline and the avoidance latencies in trial 1 were not significant difference. However, for the avoidance latencies in trial 2, the latency times of the diazepam treated Ovx rat were lower than the vehicle treated Ovx rat with the significant effect at the dosage of 0.25 mg/kg [$F(3, 38) = 3.25$, $P = 0.0332$] (Figure 4-16A, left panel). For the escape test, the latency was significantly increased only in the Ovx rats treated with diazepam at the dosage of 1.0 mg/kg compared to vehicle treated Ovx rats [$F(3, 38) = 4.45$, $P = 0.0094$] (Figure 4-16A, right panel).

The effect of benzodiazepine agonist on anxiety-like behavior in the E_2 rats as measured by the ETM are shown in figure 4-16B. The inhibitory avoidance trials from the ETM tests revealed a significant effect of dose [$F(3, 119) = 5.46$, $P = 0.0016$] and trials [$F(2, 118) = 8.41$, $P = 0.0004$]. When the comparison was made between doses of diazepam within the same trial, the baseline and the avoidance latencies in trial 1 were not significantly different. For the avoidance latencies in trial 2, although the latency times of the diazepam treated E_2 rat at the dosages of 0.25 and 0.5 mg/kg were lower than the vehicle treated E_2 rat, it was not significant difference. Additionally, the latency time of the diazepam treated E_2 rat at the dosages of 1.0

mg/kg were likely to be higher than the vehicle treated E_2 rat [F (3, 38) = 5.28, P = 0.0040] (Figure 4-16B, left panel). For the escape test, the latency tended to increase in a dose dependent manner [F (3, 38) = 2.63, P = 0.0652] (Figure 4-16B, right panel).

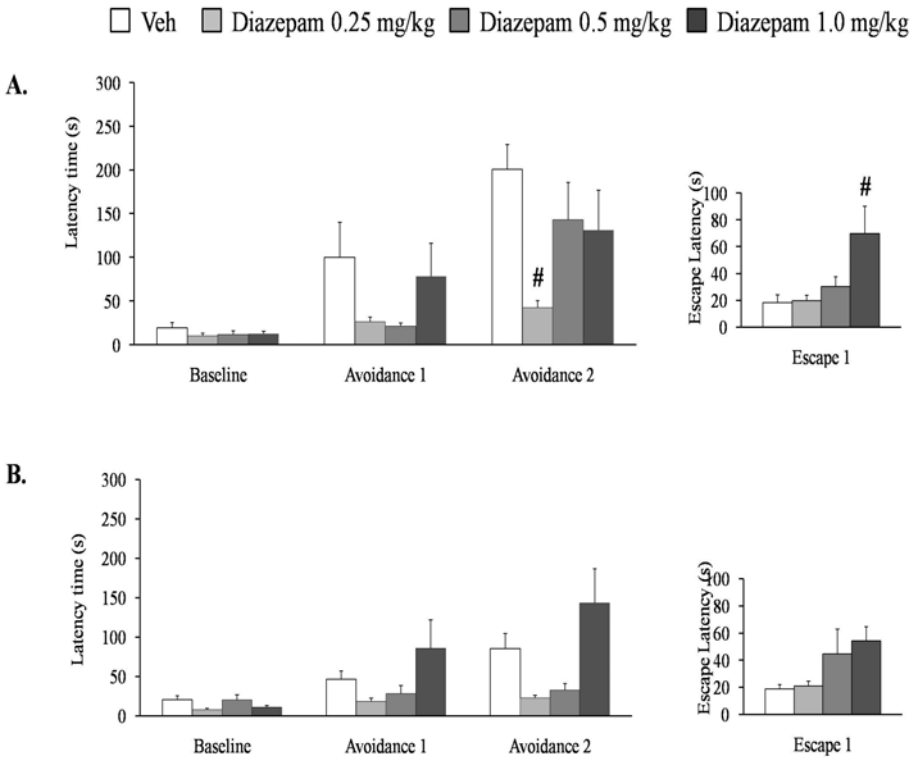


Figure 4-16 The effect of benzodiazepine agonist on anxiety-like behavior in the ETM of the (A) Ovx and (B) E_2 groups. The figures on the left panel are inhibitory avoidance trials and the figures on the right panel are escape trials. Data presented as mean $\pm$ SEM; $^{\#}$ $P<0.05$ compared to vehicle treated Ovx or E_2 rats, ANOVA followed by Dunnett's test, n= 9-10 for each subgroup.

For the locomotor activity as determined by the total number of line crossed revealed similar effect in both Ovx and E_2 rats in that the total number of line crossed in the Ovx or E_2 rats treated with diazepam at the dosage of 1 mg/kg was lower than those treated with vehicle [Ovx: F(3, 38) = 6.84, P = 0.0009; E2: F(3, 38) = 8.08, P = 0.0003] as shown in figure 4-17.

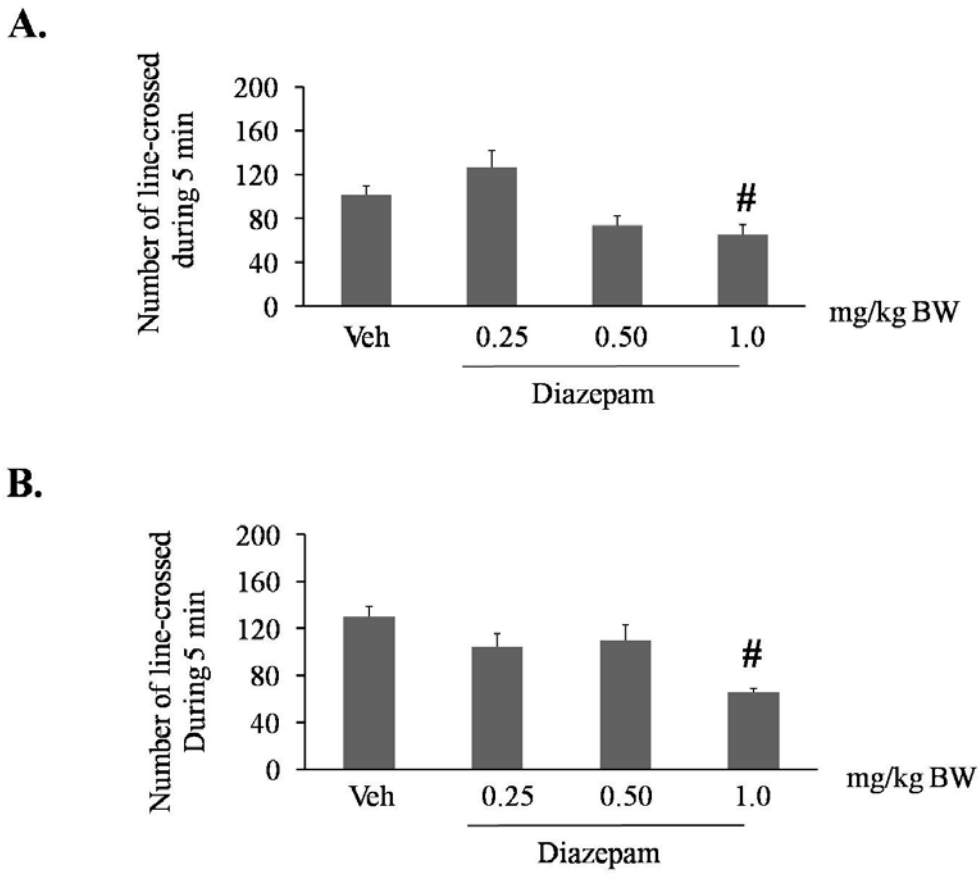


Figure 4-17 The effect of benzodiazepine agonist on locomotor activity in (A) Ovx groups and (B) E_2 groups. Data presented as mean $\pm$ SEM; $^{\#}$ P<0.05 compared to vehicle treated Ovx or E_2 rats, ANOVA followed by Dunnett's test, n= 9-10 for each subgroup.

3.2. The effects of benzodiazepine agonist on serotonergic activity in brain associated with anxiety

After behavioral test, the rat's brains were rapidly removed for measurement of 5-HT and 5-HIAA levels by HPLC technique. Figure 4-18 and 4-19 represent the example of chromatogram of 5-HT and 5-HIAA in midbrain of Ovx and E_2 rats after received various doses of benzodiazepine agonist (diazepam, 0.25, 0.50 or 1 mg/kg).

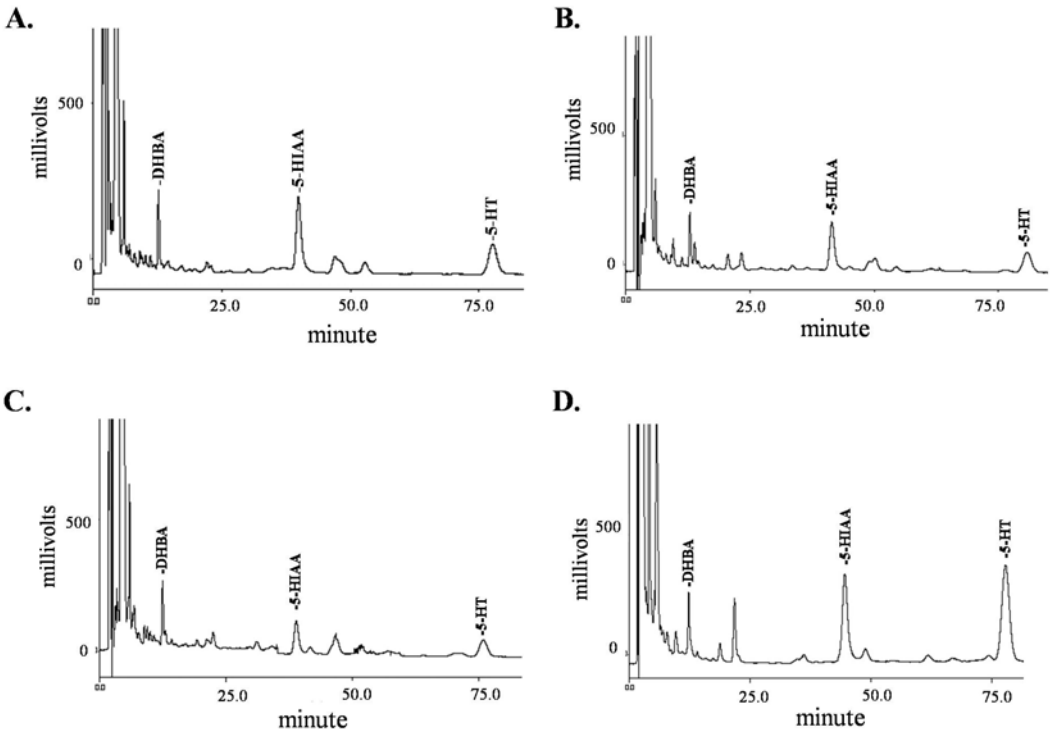


Figure 4-18 The chromatograms represent 5-HT and 5-HIAA levels in midbrain of (A) vehicle-, (B) 0.25 mg/kg diazepam-, (C) 0.5 mg/kg diazepam- and (D) 1.0 mg/kg diazepam- treated Ovx rats as measured by HPLC-EC. The retention times of 5-HIAA and 5-HT were approximately 37.75 and 75.41, respectively.

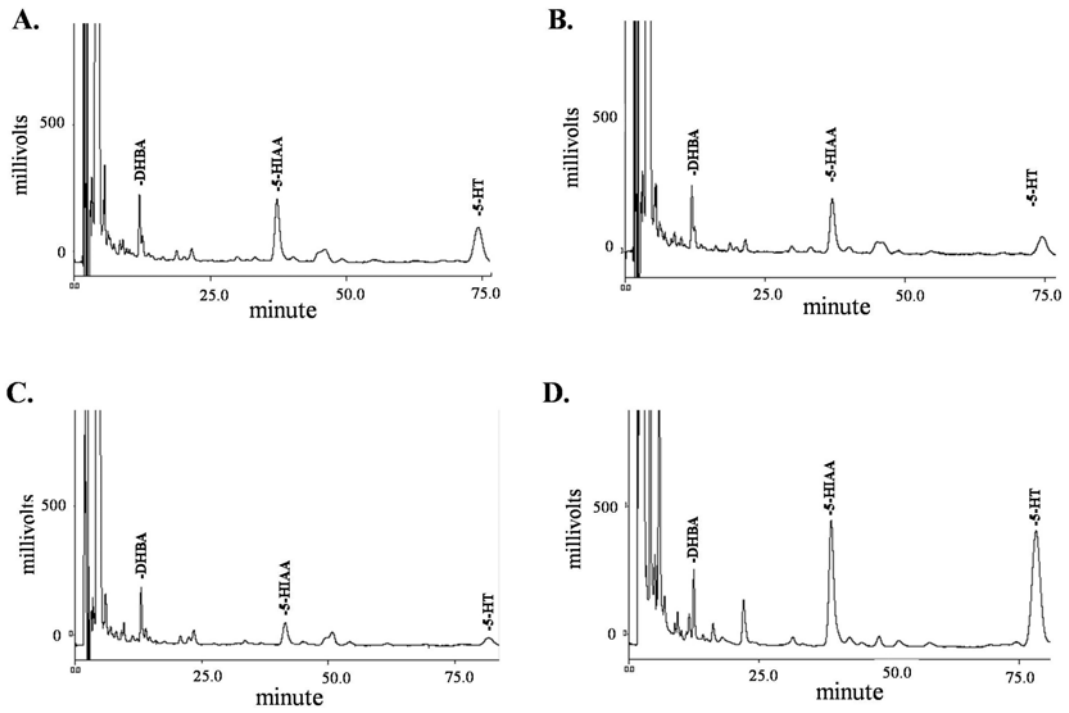


Figure 4-19 The chromatograms represent 5-HT and 5-HIAA levels in midbrain of (A) vehicle-, (B) 0.25 mg/kg diazepam-, (C) 0.5 mg/kg diazepam- and (D) 1.0 mg/kg diazepam- treated E_2 rats as measured by HPLC-EC. The retention times of 5-HIAA and 5-HT were approximately 37.75 and 75.41, respectively.

In the midbrain, two-way ANOVA revealed a significant effect of treatment for 5-HT and 5-HIAA levels (5-HT: [F (1, 51) = 13.38, P = 0.0007]; 5-HIAA: [F (1, 51) = 4.57, P = 0.0382]) with no significant effect on 5-HIAA/5-HT ratio. The levels of 5-HT and 5-HIAA in the Ovx groups were significantly lower than the E_2 groups (Figure 4-20). When the comparison was made between doses within the same group, the levels of the 5-HT and 5-HIAA and the ratio of 5-HIAA/5-HT in both the Ovx and E_2 groups were not significantly different.

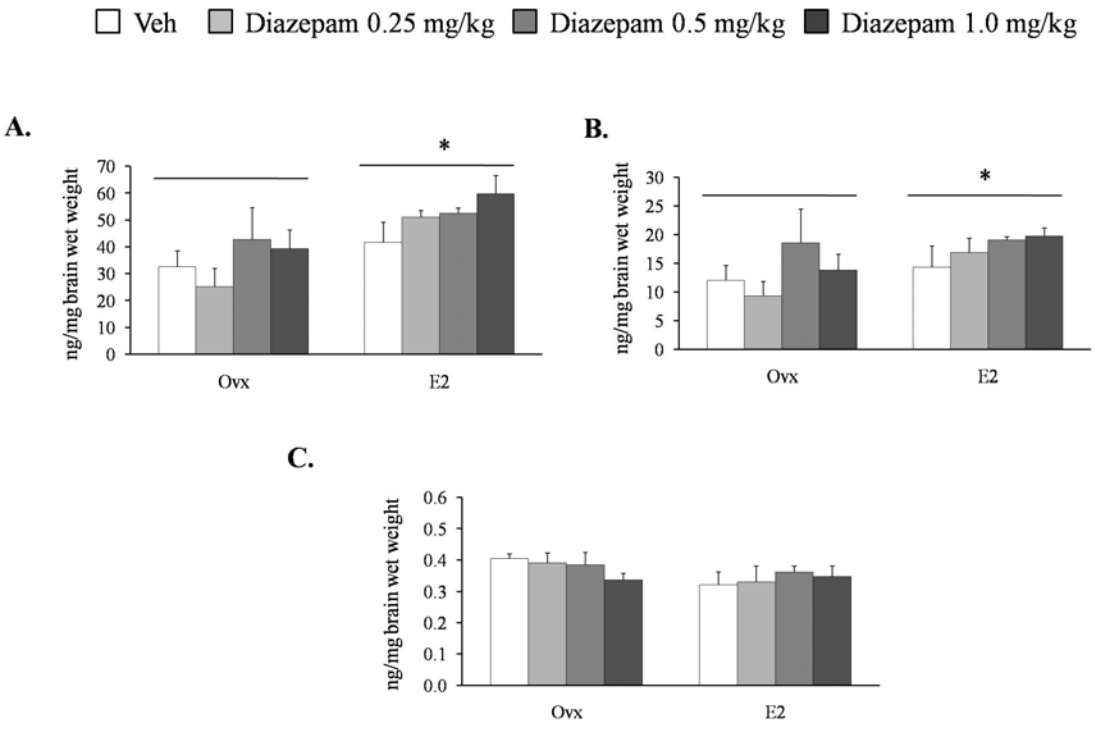


Figure 4-20 The effects of benzodiazepine agonist on (A) 5-HT, (B) 5-HIAA levels and (C) 5-HIAA/5-HT ratios in the midbrain. Data presented as mean ± SEM; * P < 0.05, significant difference from Ovx groups, two-way ANOVA. n = 6-7 for each subgroup.

In the amygdala, two-way ANOVA showed a significant effect of treatment for 5-HT and 5-HIAA levels (5-HT: [F (1, 50) = 8.67, P = 0.0052]; 5-HIAA: [F (1, 50) = 5.97, P = 0.0186]). There was no significant effect of treatment or dose on 5-HIAA/5-HT ratio. The levels of 5-HT and 5-HIAA in the Ovx groups were significant lower than the E_2 groups (Figure 4-21). When the comparison was made between doses within the same group, the levels of the 5-HT and 5-HIAA and the ratio of 5-HIAA/5-HT in both the Ovx and E_2 groups were not significantly different.

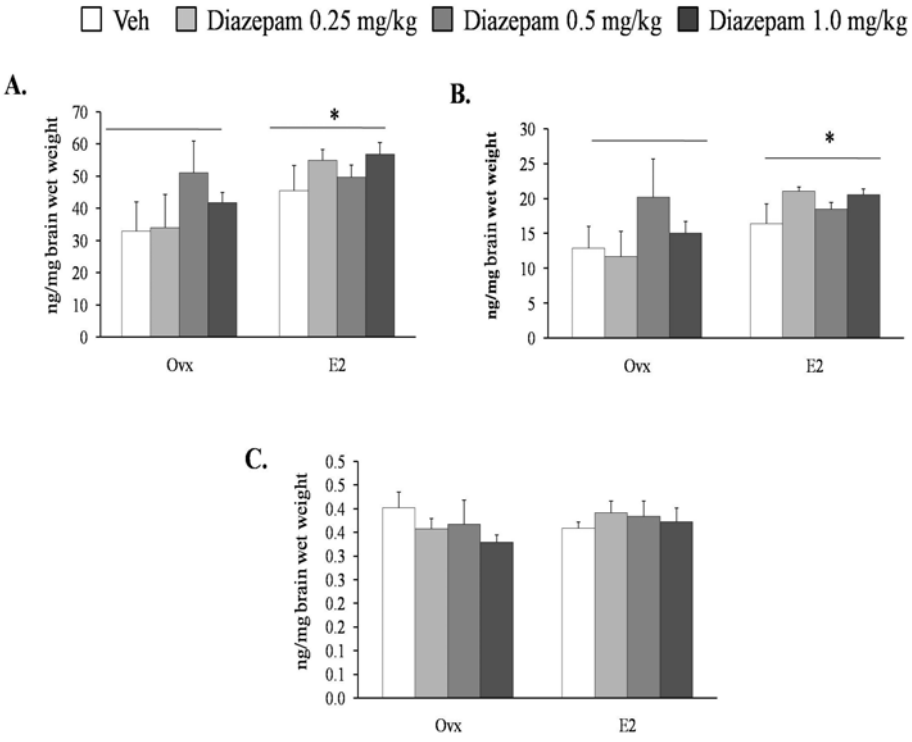


Figure 4-21 The effects of benzodiazepine agonist on (A) 5-HT, (B) 5-HIAA levels and (C) 5-HIAA/5-HT ratios in the amygdala. Data presented as mean ± SEM; * P < 0.05, significant difference from Ovx groups, two-way ANOVA. n = 6-7 for each subgroup.

In frontal cortex, two-way ANOVA revealed that there was no significant effect of treatment or dose for 5-HT level and 5-HIAA/5-HT ratio (Figure 4-22). However, the significant effect of dose for 5-HIAA levels was found [F (3, 51) = 2.91, P = 0.0448] in that the levels of 5-HIAA in the rats received diazepam were significantly higher than those that received vehicle. When the comparison was made between doses within the same group, the levels of 5-HT and 5-HIAA and the ratio of 5-HIAA/5-HT in the Ovx groups were not different. In E_2 groups, the 5-HT and 5-HIAA levels in the rats received diazepam were significantly increased compared with the rats received vehicle (5-HT: [F (3, 25) = 4.32, P = 0.0154]; 5-HIAA: [F (3, 25) = 6.51, P = 0.0025]); with significant effect at the doses of 0.25 and 1.0 mg/kg for 5-HT (Figure 4-22A) and at the doses of 0.25, 0.5 and 1.0 mg/kg for 5-HIAA (Figure 4-22 B). Consequently, there was no difference in the ratio of 5-HIAA/5-HT (Figure 4-22C).

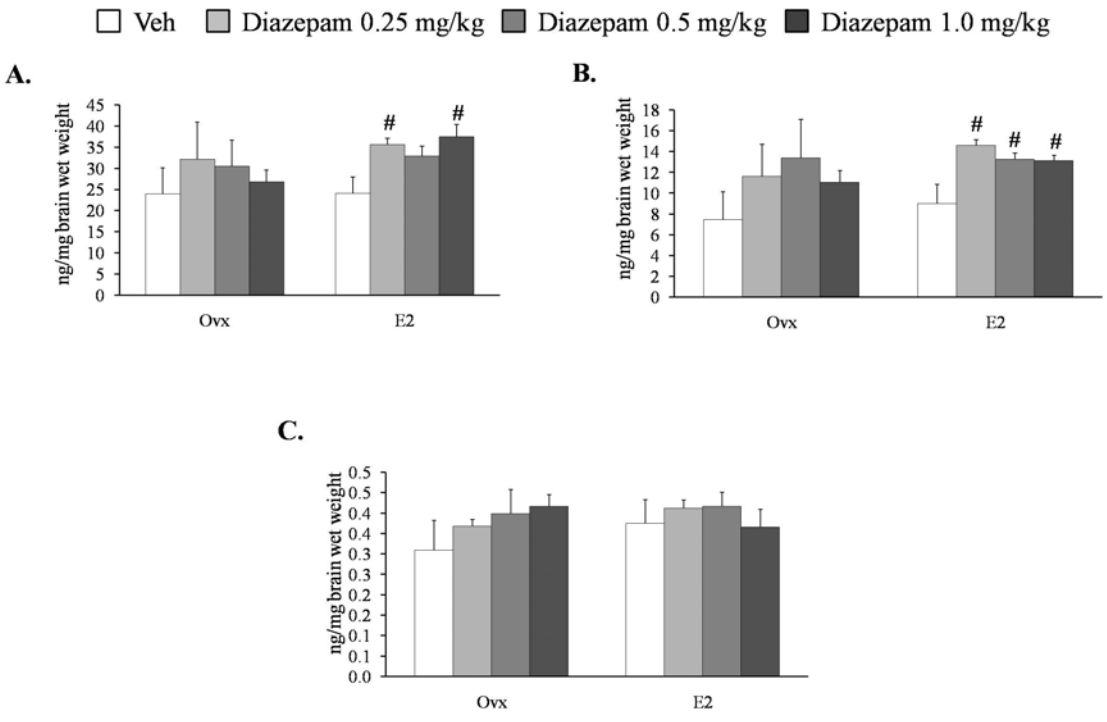


Figure 4-22 The effects of benzodiazepine agonist on (A) 5-HT, (B) 5-HIAA levels and (C) 5-HIAA/5-HT ratios in the frontal cortex. Data presented as mean ± SEM; # P < 0.05, significant difference from vehicle treated rats within the same group, ANOVA followed by Dunnett's test, n = 6-7 for each subgroup.

In hippocampus, two-way ANOVA showed a significant effect of treatment for 5-HT and 5-HIAA levels (5-HT: [F (1, 51) = 7.24, P = 0.0101]; 5-HIAA: [F (1, 51) = 14.53, P = 0.0004]), with no significant effect for 5-HIAA/5-HT ratio (Figure 4-23). The 5-HT and 5-HIAA levels in E_2 group were higher than Ovx group. When the comparison was made between doses within the same group, the levels of 5-HT and 5-HIAA and the ratio of 5-HIAA/5-HT in both the Ovx and E_2 groups were not different.

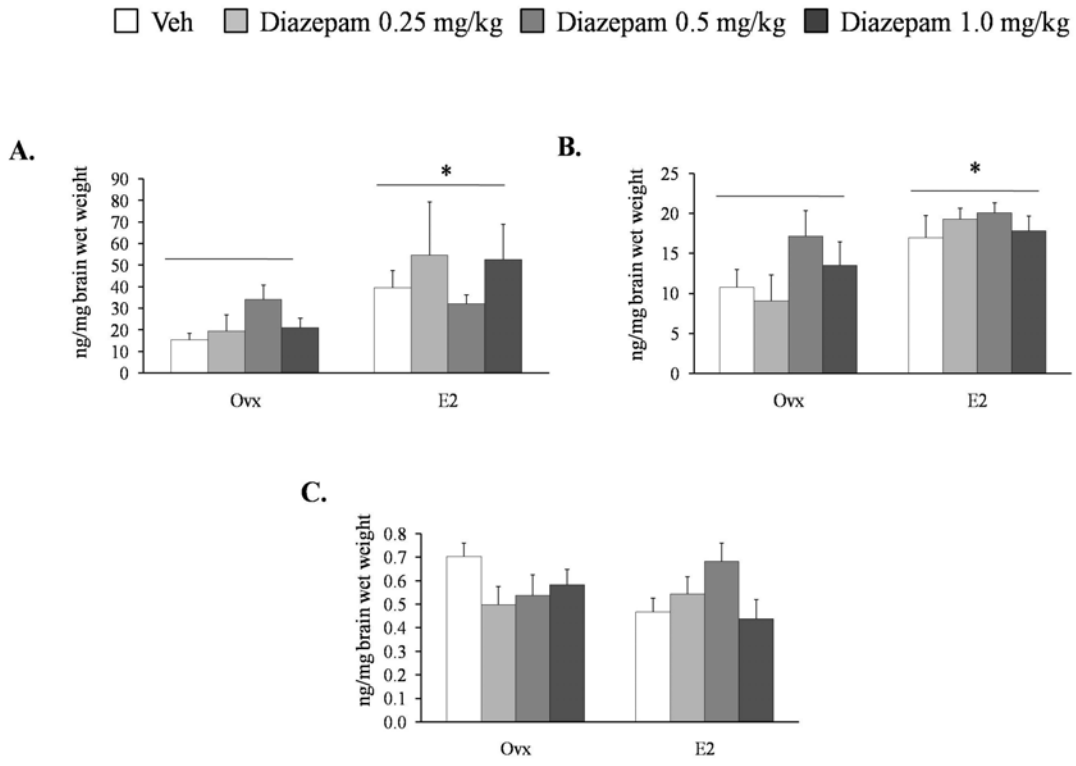


Figure 4-23 The effects of benzodiazepine agonist on (A) 5-HT, (B) 5-HIAA levels and (C) 5-HIAA/5-HT ratios in the hippocampus. Data presented as mean ± SEM; * P < 0.05, significant difference from Ovx groups, two-way ANOVA. n = 6-7 for each subgroup.

In nucleus accumbens, two-way ANOVA revealed a significant effect of treatment for 5-HT and 5-HIAA levels and 5-HIAA/5-HT ratio (5-HT: [F (1, 52) = 4.87, P = 0.0325]; 5-HIAA: [F (1, 52) = 7.96, P = 0.0071]; 5-HIAA/5-HT: [F (1, 52) = 10.79, P = 0.0020]). The levels of 5-HT and 5-HIAA levels and 5-HIAA/5-HT ratio in the E_2 group were higher than the Ovx group (Figure 4-24). When the comparison was made between doses within the same group, the levels of 5-HT and 5-HIAA and the ratio of 5-HIAA/5-HT in the Ovx group were not different. In the E_2 groups, the levels of 5-HT and 5-HIAA/5-HT ratio were not significantly different; however, the level of 5-HIAA in the rat received diazepam was higher than those received vehicle with significant difference at the dose of 0.5 mg/kg (Figure 4-24B).

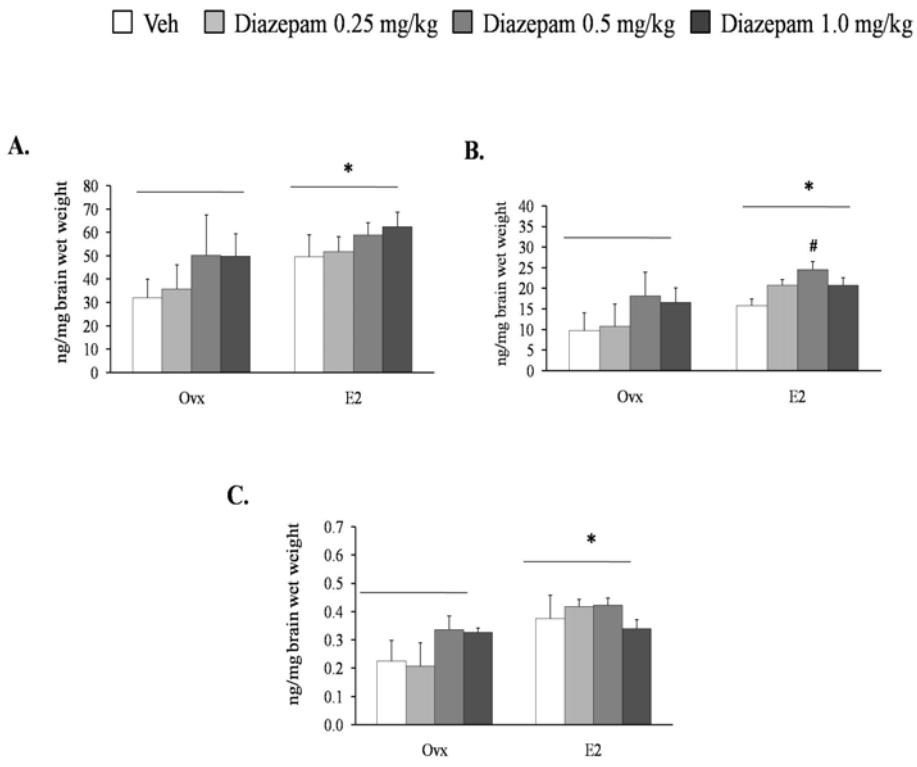


Figure 4-24 The effects of benzodiazepine agonist on (A) 5-HT, (B) 5-HIAA levels and (C) 5-HIAA/5-HT ratios in the nucleus accumbens. Data presented as mean ± SEM; * $P < 0.05$, significant difference from Ovx groups, two-way ANOVA. n = 6-7 for each group. $^{\#}P < 0.05$, significant difference from vehicle treated rats within the same group, ANOVA followed by Dunnett's test, n = 6-7 for each subgroup.

In the septum, there was no significant effect of treatment or dose for 5-HT and 5-HIAA levels; however, a significant effect of treatment for 5-HIAA/5-HT ratio was found [F (1, 50) = 9.44, P = 0.0037] (Figure 4-25). The ratio of 5-HIAA/5-HT in the E_2 group was higher than the Ovx group (Figure 4-25C). When the comparison was made between doses within the same group, the levels of 5-HT and 5-HIAA and the ratio of 5-HIAA/5-HT in both the Ovx and the E_2 groups were not different.

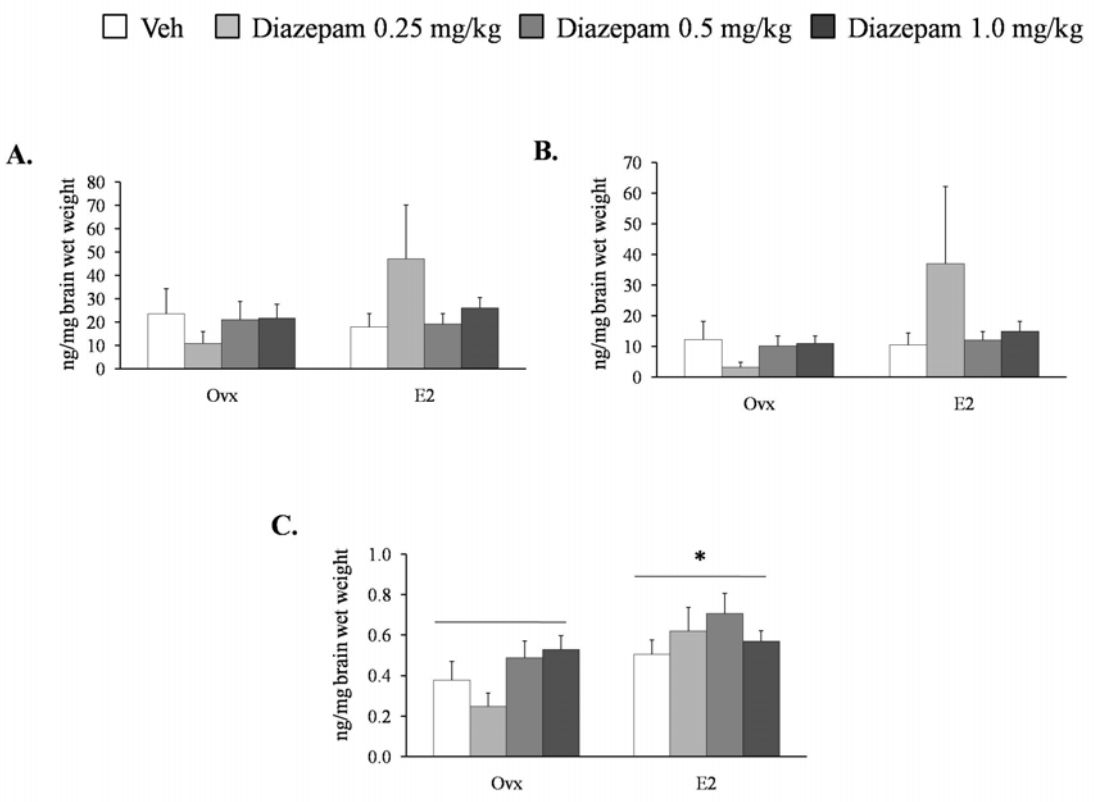


Figure 4-25 The effects of benzodiazepine agonist on (A) 5-HT, (B) 5-HIAA levels and (C) 5-HIAA/5-HT ratios in the septum. Data presented as mean ± SEM; * P < 0.05, significant difference from Ovx groups, two-way ANOVA. n = 6-7 for each group.

In anterior hypothalamus, two-way ANOVA showed no significant effect of treatment or dose for 5-HT level but a significant effect of treatment for 5-HIAA level and 5-HIAA/5-HT ratio were found (5-HIAA: [F (1, 51) = 5.47, P = 0.0240]; 5-HIAA/5-HT: [F (1, 51) = 7.17, P = 0.0105]) (Figure 4-26). The 5-HIAA level and 5-HIAA/5-HT ratio of E_2 group were significantly higher than the Ovx group. When the comparison was made between doses within the same group, the levels of 5-HT and 5-HIAA and the ratio of 5-HIAA/5-HT in neither the Ovx groups nor the E2 group were not different between doses.

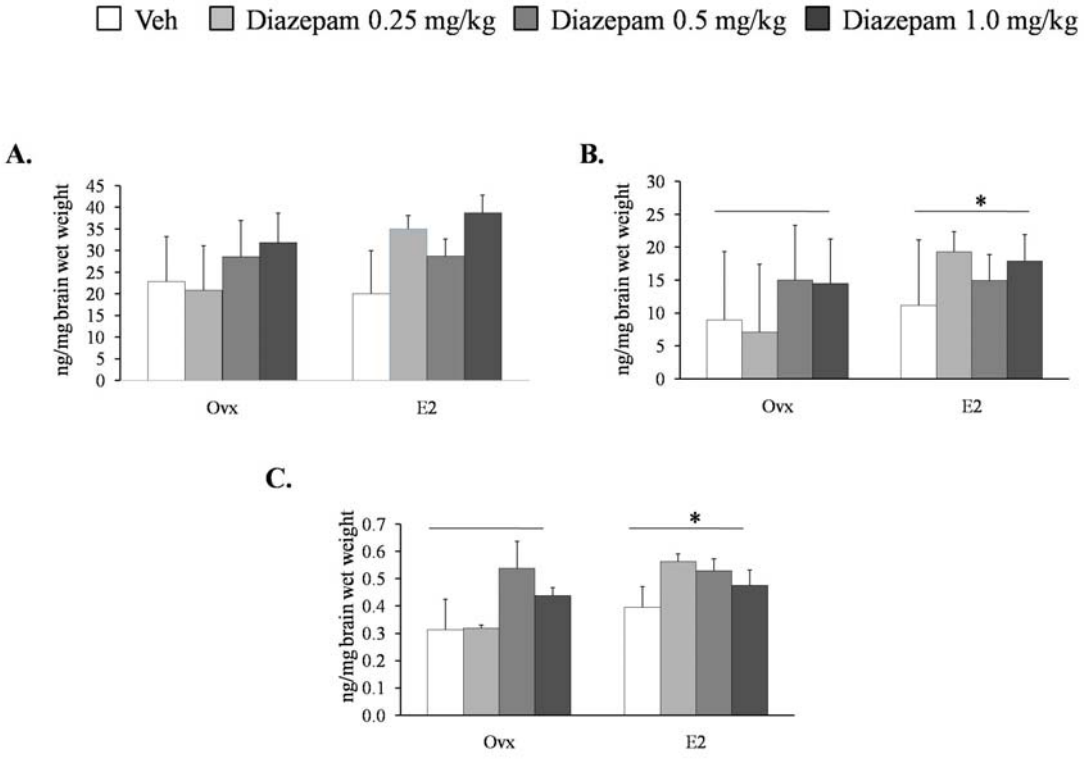


Figure 4-26 The effects of benzodiazepine agonist on (A) 5-HT, (B) 5-HIAA levels and (C) 5-HIAA/5-HT ratios in the anterior hypothalamus. Data presented as mean $\pm$ SEM; * $P < 0.05$, significant difference from Ovx groups, two-way ANOVA. n = 6-7 for each group.

Part3: To investigate whether estrogen can alleviate anxiety-like behavior in ovariectomized rat and investigate 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 part, female Wistar rats were ovariectomized for 21 day to induce anxiety as it had been shown in experiment 1 that this period was enough to induce anxiety; thereafter, the rats were divided into 2 groups, Ovx and E_2 to received vehicle or 17β-estradiol for 28 days. At the end of experiment, the rats from each group were randomly selected to inject with benzodiazepine agonist (diazepam, 0.25 mg/kg) 30 min before behavioral test. This dose of diazepam was selected based on the results from the experiment 2 that it contained anxiolytic-like effect in the Ovx rats with no effect on locomotor activity. Body weight and food intake were recorded daily. A uterus in all groups of animals was weighed immediately after sacrifice.

3.1 The effects of estrogen on body weight, food intake and uterine weight after long-term ovariectomy

Table 4-3 summarizes the mean values $\pm$ S.E.M. of the physiological data including body weight and daily food intake at different periods of experiment, uterine weight at the end of the experiment, the calculated percentage change of body weight and percentage of uterine weight to body weight of Ovx and E_2 groups.

The body weights of the rats between the Ovx and E_2 groups at the beginning of the experiment and at 21 days after ovariectomy were not different. The percentage change of body weight at 21 days after ovariectomy was thus not differed (Table 4-3). After estrogen administration for 28 days, the E_2 treated rat demonstrated a significant reduction in body weight [$t(38) = 3.77$, $P = 0.0006$] and the percentage change of body weight [$t(38) = 6.70$, $P < 0.0001$]. After estrogen administration for 28 days, the daily food intake was also lower in the E_2 group but not statistically significant difference from the Ovx group.

A significant increase in uterine weight [t(38) = 7.83, P < 0.0001] and percentage of uterine weight to body weight [t(38) = 7.79, P < 0.0001] was demonstrated in E_2 group.

Table 4-3 The body weight, the percentage change of body weight, the daily food intake, the uterine weight and the percentage of uterine weight to body weight in Ovx and E_2 rats.

Parameters	**Ovx**	**E_2**
Body weight (gm)		
start of experiment	211.00 ± 2.94	209.50 ± 2.60
21 days after ovariectomy	273.25 ± 3.56	280.25 ± 2.70
End of experiment	300.13 ± 5.21	276.88 ± 3.31*
Body weight change (%)		
21 days after ovariectomy	32.17 ± 1.86	34.12 ± 1.94
28 days after vehicle/ E_2 supplementation	7.81 ± 1.01	-1.18 ± 0.89**
Daily Food Intake (gm/d)		
21 days after ovariectomy	16.93 ± 0.44	16.95 ± 0.29
28 days after vehicle/E_2 supplementation	15.81 ± 0.67	14.40 ± 0.31
Uterine Weight (gm)	0.135 ± 0.006	0.311 ± 0.006**
Uterine Weight to Body Weight (%)	0.046 ± 0.006	0.113 ± 0.006**

Data presented as mean ± S.E.M., *P<0.005 and **P<0.0001, significantly different from corresponding Ovx group using Student's unpaired *t*-test. n = 10 to each subgroup.

3.2 The effects of benzodiazepine agonist on anxiety-like behavior after long-term ovariectomy

The effects of benzodiazepine agonist on anxiety-like behavior after long-term ovariectomy (49 days) as measured by the ETM are shown in the figure 4-27. After 49 day of ovariectomy, the avoidance latency in trial 2 of the Ovx rat was longer than the E_2 group, indicating anxiety-like behavior of the Ovx rats [$t(18) = 4.61$, $P = 0.0002$] (Figure 4-27A and B). In order to determine the alteration of $GABA_A$ receptor function after long-term ovariectomy, the comparison was made with in the same group i.e. the Ovx or E_2 group. After diazepam administration (0.25 mg/kg), the avoidance latency in trial 2 of the Ovx group was shorter than vehicle treated-counterpart [$t (18) = 2.71$, $P = 0.0144$] with no change in neither the baseline nor the avoidance latency in trial 1. On the other hand, in the E_2 group, the diazepam had no effect on neither the baseline nor the avoidance latencies in trial 1 and 2 ($P > 0.05$).

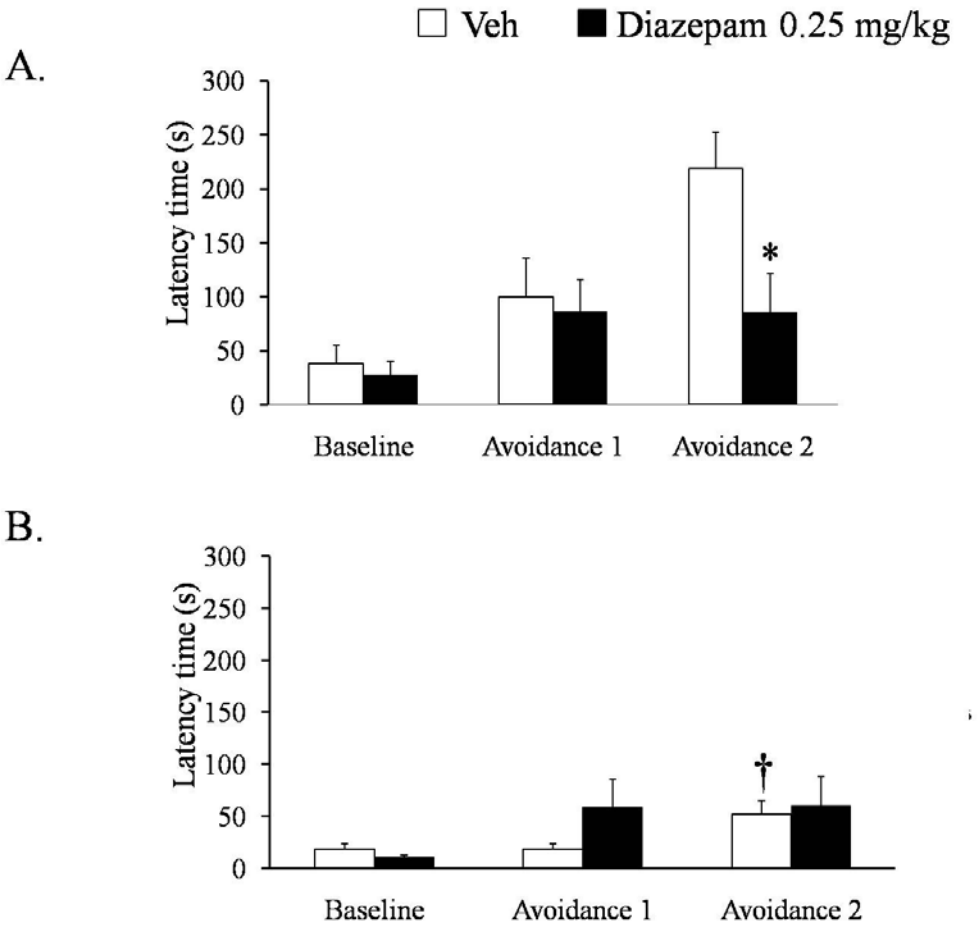


Figure 4-27 The effects of benzodiazepine agonist on anxiety-like behavior after long-term ovariectomy in (A) Ovx and (B) E_2 rats. Data presented as mean ± S.E.M., * $P < 0.05$, compared with vehicle treated rats within the same group; † $P < 0.05$ compared between Ovx and E_2 group with the same treatment i.e. vehicle- or diazepam-treated rats; using Student's unpaired *t*-test. n = 9-10 to each subgroup.

For the escape latency, there was no significant difference between Ovx and E_2 or the vehicle- and diazepam- treated rats ($P > 0.05$) (Figure 4-28A). The locomotor activity as determined by the total number of line crossed in the open field during 5 min was not differed between between Ovx and E_2 or the vehicle- and diazepam- treated rats ($P > 0.05$) (Figure 4-21B), suggesting that the diazepam at the dosage of 0.25 mg/kg had no effect on locomotor activity.

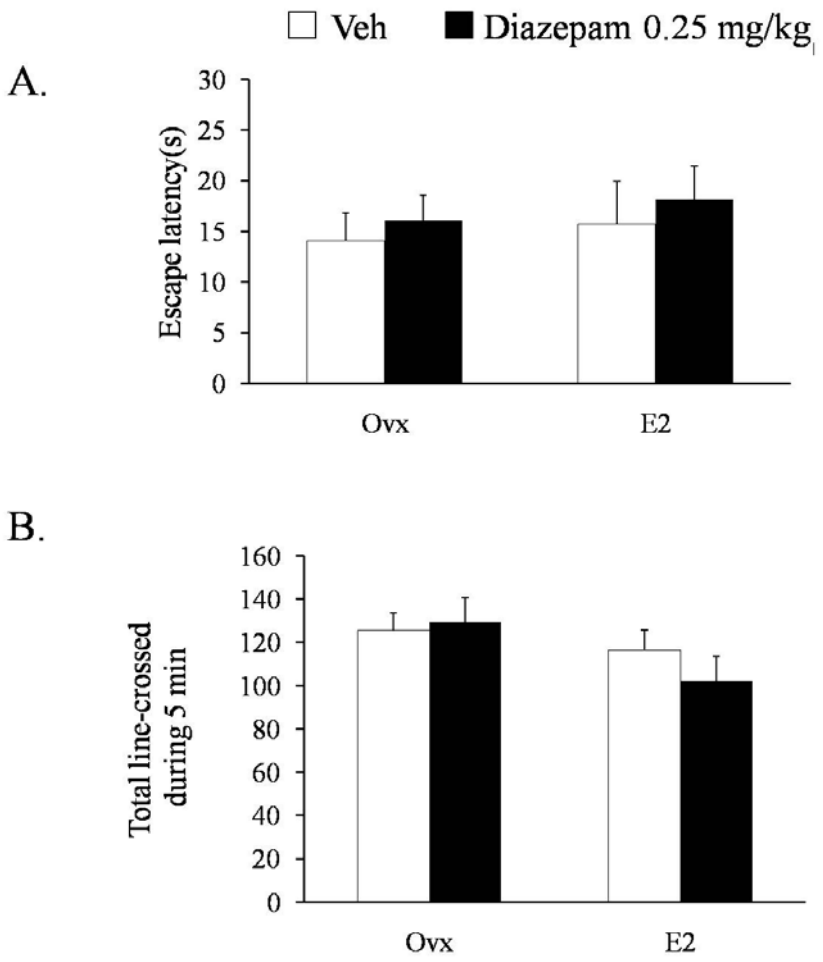


Figure 4-28 The effects of benzodiazepine agonist on (A) escape behavior and (B) locomotor activity in the Ovx and E_2 rats after long-term ovariectomy. Data presented as mean $\pm$ S.E.M. n = 9-10 to each subgroup.

3.3 The effects of benzodiazepine agonist on serotonergic activity in brain areas related to anxiety after long-term ovariectomy

The effects of benzodiazepine agonist (diazepam) administration on serotonergic activity in brain areas contributing to anxiety in Ovx and E_2 rats are presented in figure 4-29.

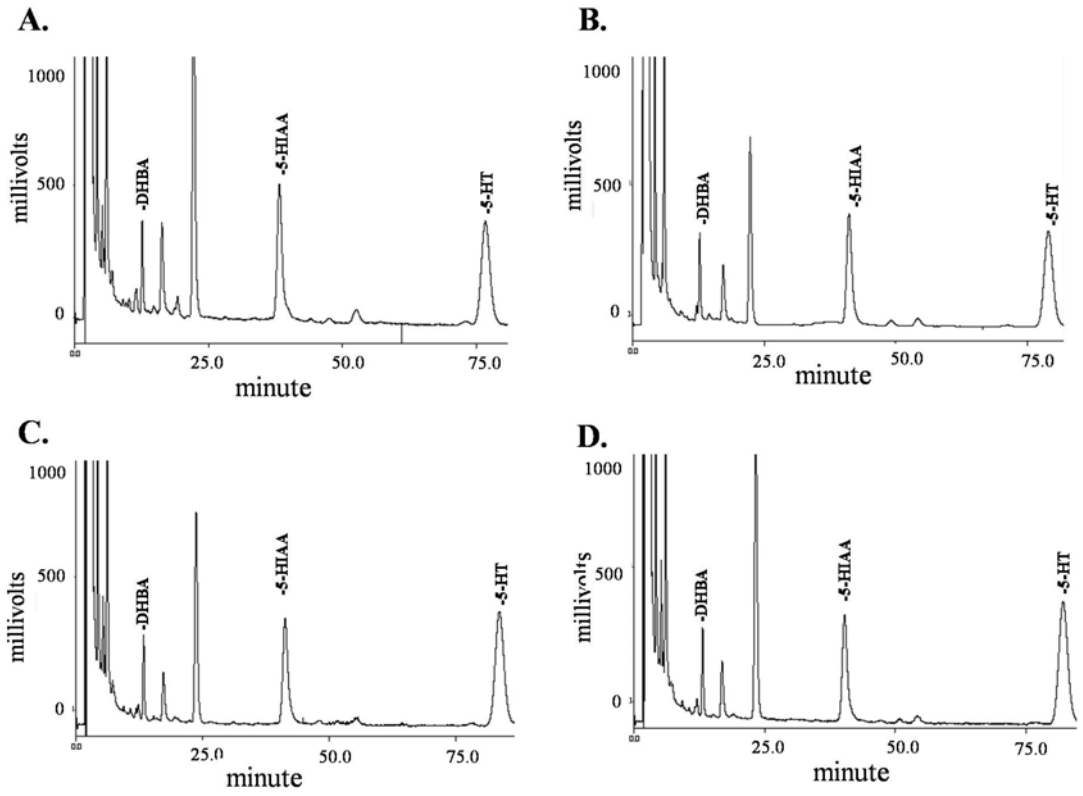


Figure 4-29 The chromatograms represent 5-HT and 5-HIAA levels in midbrain of (A) Ovx rats treated with vehicle, (B) Ovx rats treated with diazepam, (C) E_2 rats treated with vehicle and (D) E_2 rats treated with diazepam as measured by HPLC-EC. The retention times of 5-HIAA and 5-HT levels were approximately 37.75 and 75.41, respectively.

The levels of 5-HT, 5-HIAA and the ratio of 5-HIAA/5-HT in the midbrain of Ovx- and E_2- groups treated with vehicle or diazepam (0.25 mg/kg) are shown in figure 4-30. The comparison between the Ovx and the E_2 groups (treated with vehicle) demonstrated that the level of 5-HT in the E_2 group tended to be lower than Ovx group [t(14) = 1.98, P = 0.0673] (Figure 4-30A) while the level of 5-HIAA and 5-HIAA/5-HT ratio were not affected. When the comparison was made between vehicle- and diazepam treated rats within the same group, the 5-HT and 5-HIAA levels and 5-HIAA/5-HT ratio in the Ovx group were not different. However, in the E_2 groups, the 5-HT level in the rats injected with diazepam (0.25 mg/kg) was significantly higher than those injected with vehicle [t(14) = 3.00, P = 0.0095] (Figure 4-30A) with no difference in the 5-HIAA level and the 5-HIAA/5-HT ratio (Figure 4-30B and C).

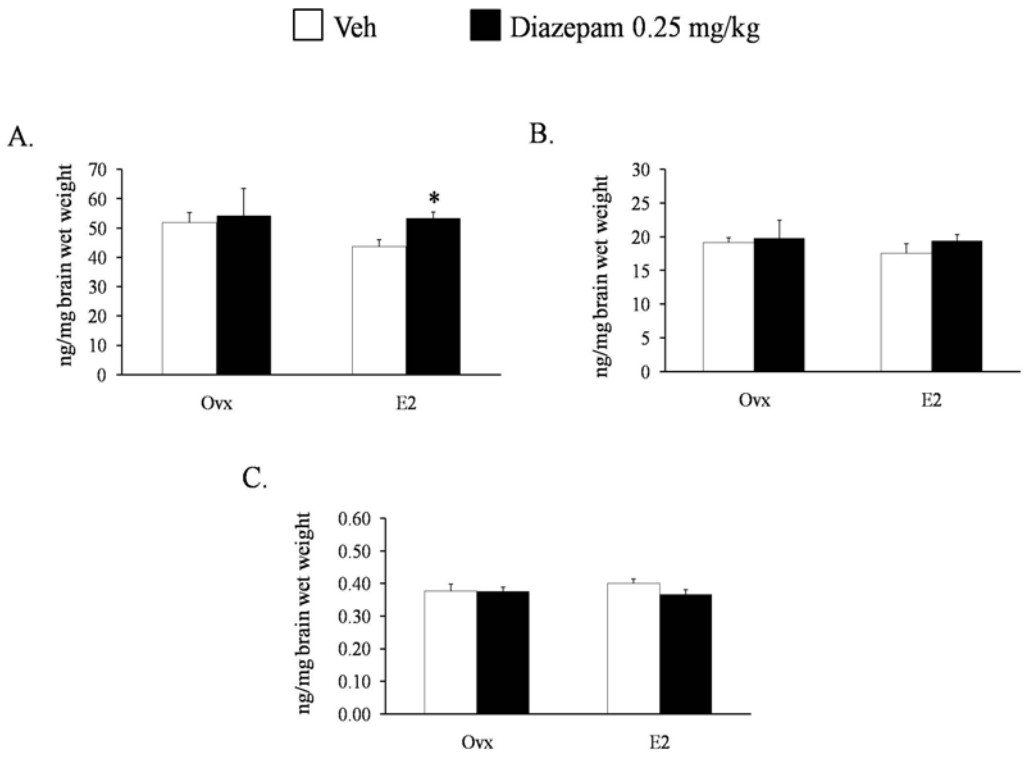


Figure 4-30 The effects of benzodiazepine agonist on the levels of (A) 5-HT, (B) HIAA and 5-HIAA/5-HT ratio in midbrain. * P<0.05, significantly different from vehicle treated rats within the same group using Student's unpaired *t*-test. n = 8 to each subgroup.

The levels of 5-HT, 5-HIAA and the ratio of 5-HIAA/5-HT in the amygdala of Ovx- and E_2- groups treated with vehicle or diazepam (0.25 mg/kg) are shown in figure 4-31. The comparison between the Ovx and the E_2 groups (treated with vehicle) demonstrated that the level of 5-HIAA in the E_2 group tended to be lower than Ovx group [t(14) = 1.94, P = 0.0731] (Figure 4-31B) while the level of 5-HT and 5-HIAA/5-HT ratio were not affected. When the comparison was made between vehicle- and diazepam treated rats within the same group, the 5-HT and 5-HIAA levels and 5-HIAA/5-HT ratio in neither the Ovx nor E_2 group were not different.

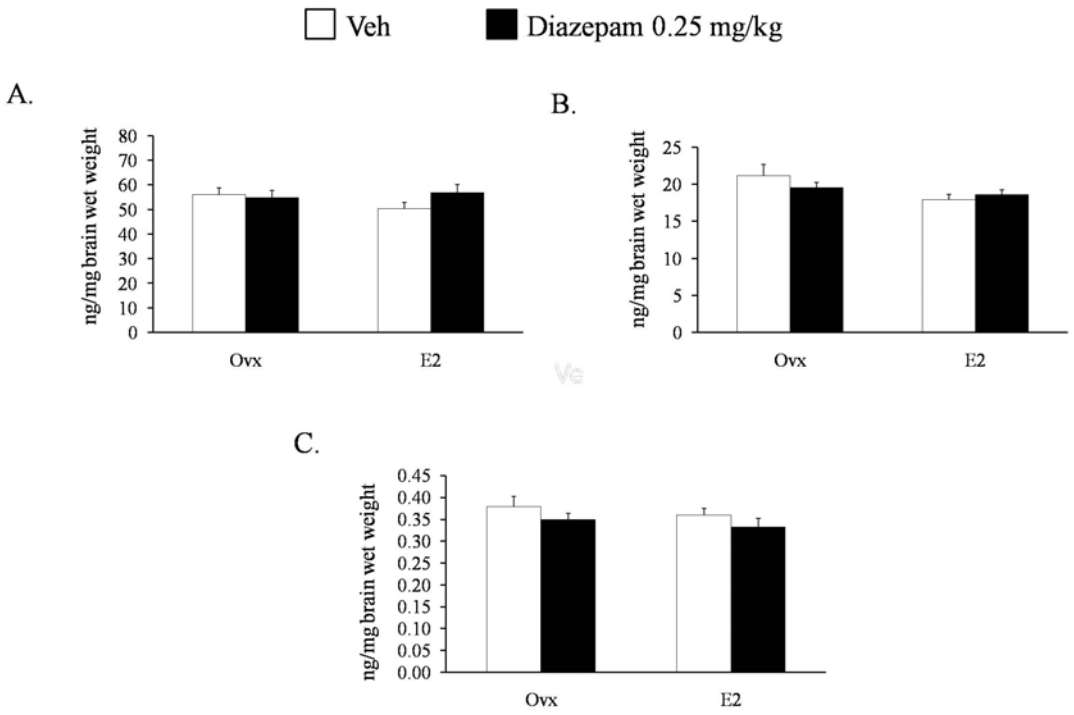


Figure 4-31 The effects of benzodiazepine agonist on the levels of (A) 5-HT, (B) HIAA and 5-HIAA/5-HT ratio in amygdala. n = 8 to each group.

The levels of 5-HT, 5-HIAA and the ratio of 5-HIAA/5-HT in the frontal cortex of Ovx- and E_2- groups treated with vehicle or diazepam (0.25 mg/kg) are shown in figure 4-32. The comparison between the Ovx and the E_2 groups (treated with vehicle) demonstrated that the levels of 5-HT, 5-HIAA and 5-HIAA/5-HT ratio were not differed. When the comparison was made between vehicle- and diazepam treated rats within the same group, the 5-HT and 5-HIAA levels and 5-HIAA/5-HT ratio in neither the Ovx nor E_2 group were not different.

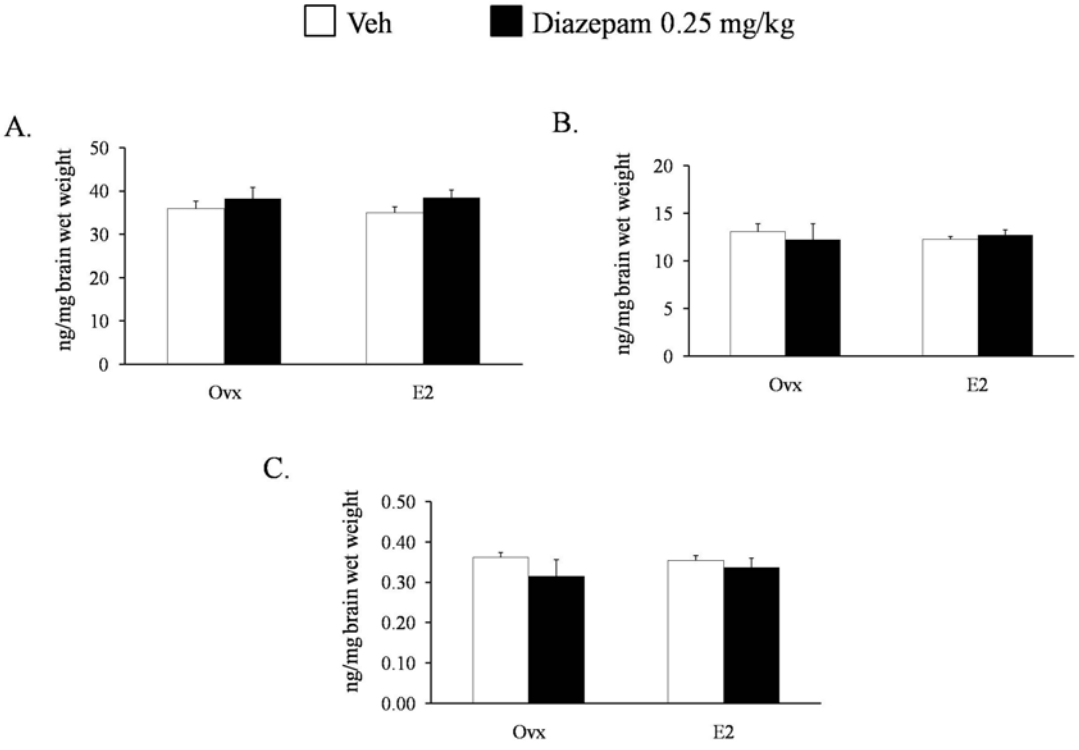


Figure 4-32 The effects of benzodiazepine agonist on the levels of (A) 5-HT, (B) HIAA and 5-HIAA/5-HT ratio in frontal cortex. n = 8 to each subgroup.

The levels of 5-HT, 5-HIAA and the ratio of 5-HIAA/5-HT in the hippocampus of Ovx- and E_2- groups treated with vehicle or diazepam (0.25 mg/kg) are shown in figure 4-33. The comparison between the Ovx and the E_2 groups (treated with vehicle) demonstrated that the level of 5-HT in the E_2 group tended to be lower than Ovx group [t (14) = 1.81, P = 0.0938] (Figure 4-33A) while the level of 5-HIAA and 5-HIAA/5-HT ratio were not affected. When the comparison was made between vehicle- and diazepam treated rats within the same group, the 5-HT and 5-HIAA levels and 5-HIAA/5-HT ratio in the Ovx group were not different. However, in the E_2 groups, the 5-HT level in the rats injected with diazepam (0.25 mg/kg) was significantly higher than those injected with vehicle [t(14) = 2.43, P = 0.0302] (Figure 4-33A) with no difference in the 5-HIAA level (Figure 4-33B). Consequently, the 5-HIAA/5-HT ratio of the diazepam- treated rats was lower than vehicle- treated rats [t(14) = 2.61, P = 0.0217] (Figure 4-33C).

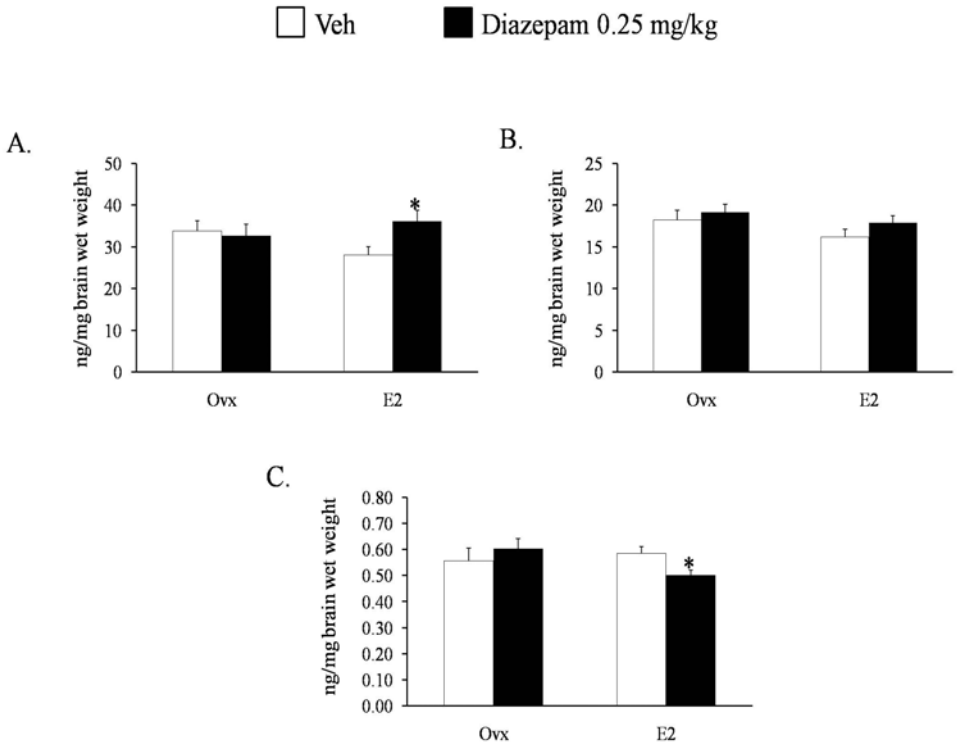


Figure 4-33 The effects of benzodiazepine agonist on the levels of (A) 5-HT, (B) HIAA and 5-HIAA/5-HT ratio in hippocampus. * P<0.05, significantly different from vehicle treated rats within the same group using Student's unpaired *t*-test. n = 8 to each subgroup.

The levels of 5-HT, 5-HIAA and the ratio of 5-HIAA/5-HT in the nucleus accumbens of Ovx- and E_2- groups treated with vehicle or diazepam (0.25 mg/kg) are shown in figure 4-34. The comparison between the Ovx and the E_2 groups (treated with vehicle) demonstrated that the levels of 5-HT, 5-HIAA and 5-HIAA/5-HT ratio were not differed. When the comparison was made between vehicle- and diazepam treated rats within the same group, the 5-HT and 5-HIAA levels and 5-HIAA/5-HT ratio in neither the Ovx nor E_2 group were not different.

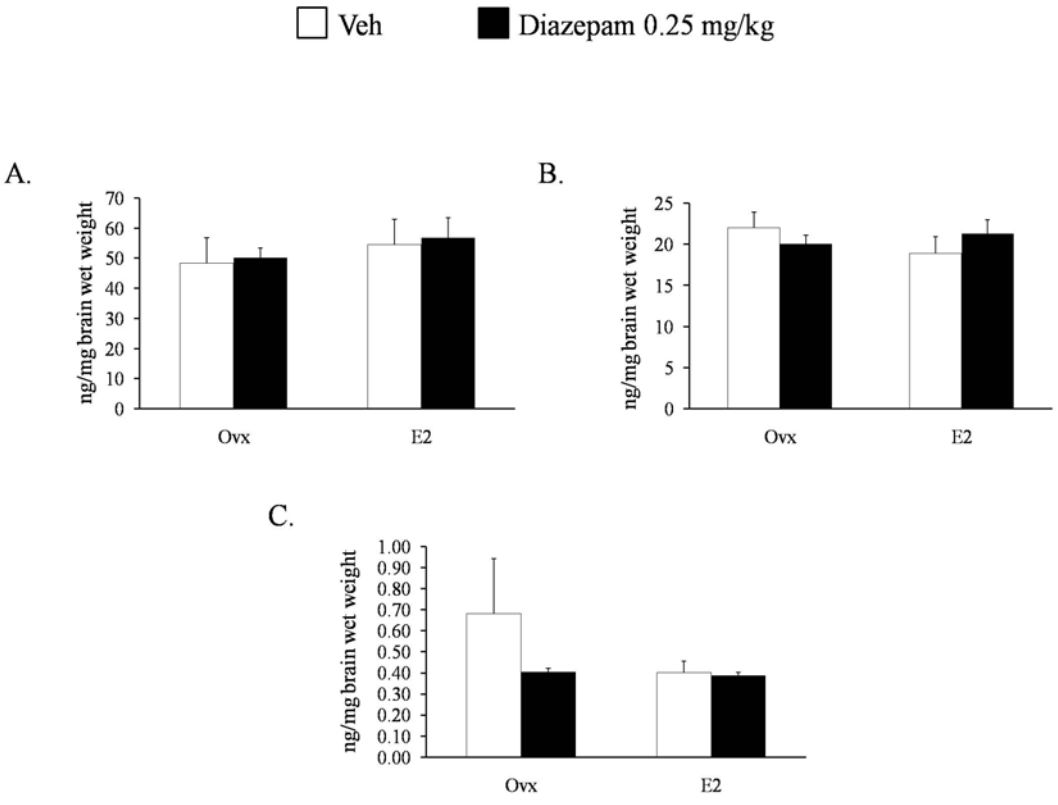


Figure 4-34 The effects of benzodiazepine agonist on the levels of (A) 5-HT, (B) HIAA and 5-HIAA/5-HT ratio in nucleus accumbens. n = 8 to each subgroup.

The levels of 5-HT, 5-HIAA and the ratio of 5-HIAA/5-HT in the septum of Ovx- and E_2- groups treated with vehicle or diazepam (0.25 mg/kg) are shown in figure 4-35. The comparison between the Ovx and the E_2 groups (treated with vehicle) demonstrated that the levels of 5-HT, 5-HIAA and 5-HIAA/5-HT ratio in the E_2 group were not different from those of Ovx group. When the comparison was made between vehicle- and diazepam treated rats within the same group, the 5-HT and 5-HIAA levels and 5-HIAA/5-HT ratio in the Ovx group were not different. However, in the E_2 groups, the 5-HIAA/5-HT ratio of the diazepam- treated rats was lower than vehicle- treated rats [t(14) = 2.99, P = 0.0098] (Figure 4-35C) with no difference in the 5-HT or 5-HIAA level (Figure 4-35A and B).

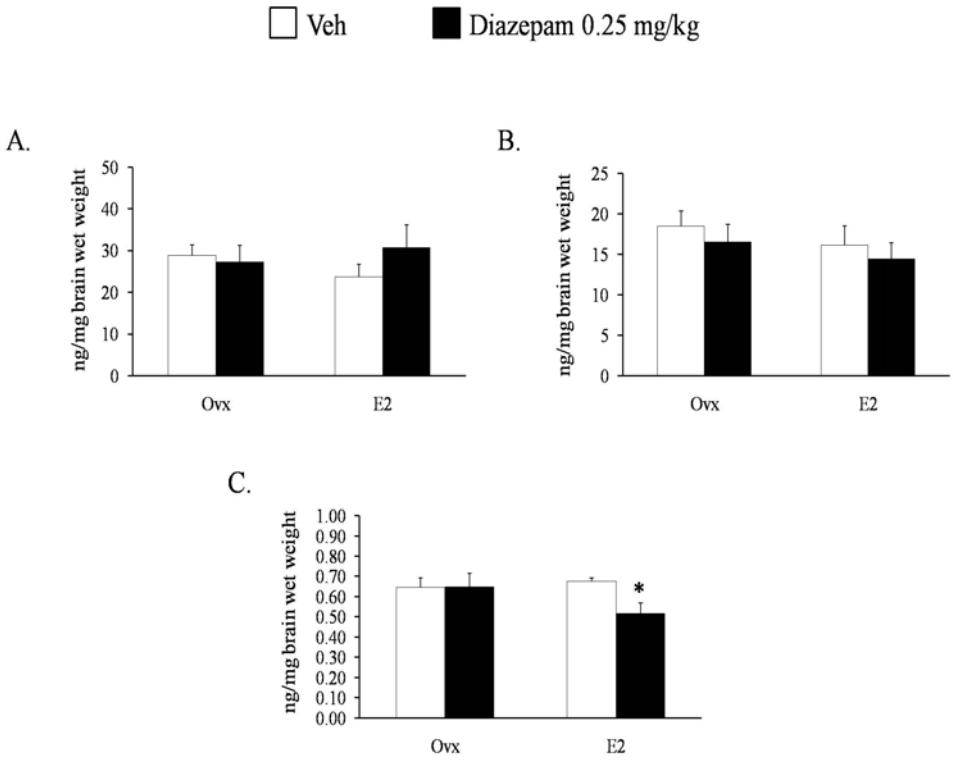


Figure 4-35 The effects of benzodiazepine agonist on the levels of (A) 5-HT, (B) HIAA and 5-HIAA/5-HT ratio in septum. * P<0.05, significantly different from vehicle treated rats within the same group using Student's unpaired *t*-test. n = 8 to each subgroup.

The levels of 5-HT, 5-HIAA and the ratio of 5-HIAA/5-HT in the anterior hypothalamus of Ovx- and E_2- groups treated with vehicle or diazepam (0.25 mg/kg) are shown in figure 4-36. The comparison between the Ovx and the E_2 groups (treated with vehicle) demonstrated that the levels of 5-HT, 5-HIAA and 5-HIAA/5-HT ratio in the E_2 group were not different from those of Ovx group. When the comparison was made between vehicle- and diazepam treated rats within the same group, the 5-HT and 5-HIAA levels and 5-HIAA/5-HT ratio in the Ovx group were not different. However, in the E_2 groups, the 5-HIAA/5-HT ratio of the diazepam-treated rats was lower than vehicle- treated rats [t(14) = 2.58, P = 0.0220] (Figure 4-36C) with no difference in the 5-HT or 5-HIAA level (Figure 4-36A and B).

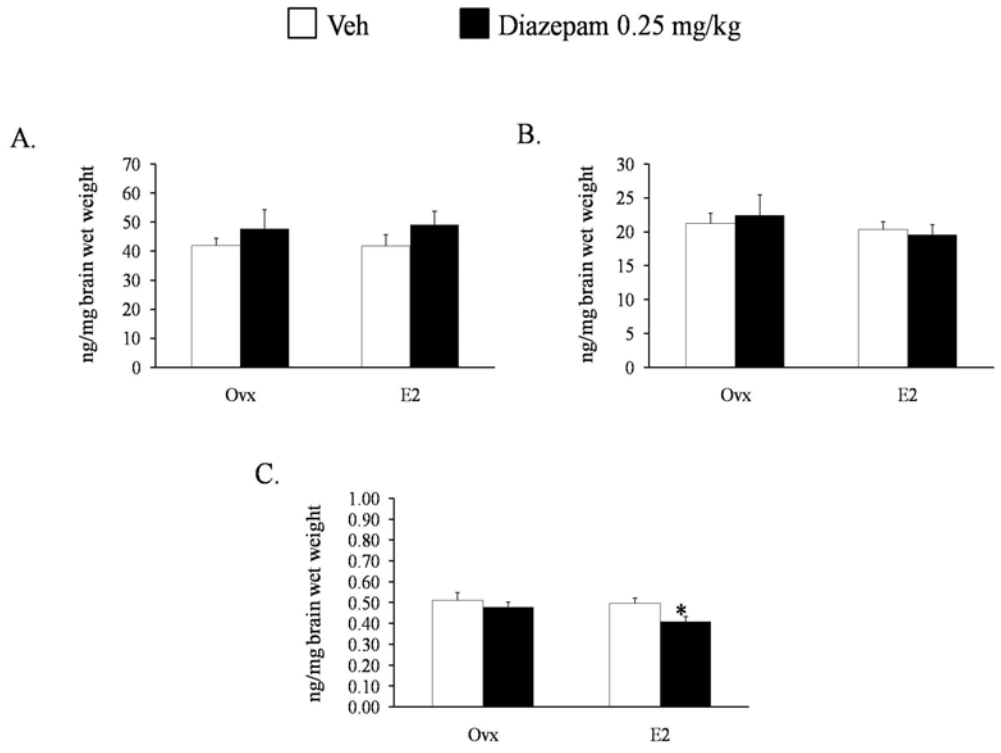


Figure 4-36 The effects of benzodiazepine agonist on the levels of (A) 5-HT, (B) HIAA and 5-HIAA/5-HT ratio in anterior hypothalamus. * P<0.05, significantly different from vehicle treated rats within the same group using Student's unpaired *t*-test. n = 8 to each subgroup.

CHAPTER V
DISCUSSION

In the present study, we hypothesized that the length of estrogen deprivation had a negative effect on the level of anxiety-like behavior measured with the ETM; and this effect may be related to the alteration of $GABA_A$ subunit expressions or functions in the brain. The alteration of $GABA_A$ receptor may alter serotonergic activity; thereby increase anxiety-like behavior in ovariectomized rats.

The effects of estrogen deprivation on body weight and food intake

In the part 1 of the current study, the rats were ovariectomized and received vehicle or estrogen (1 μg/kg) for different period of time ranging from 1 to 4 weeks. The results showed that the body weight and body weight gain of Ovx rats were higher than E_2 rats at all time point; while the daily food intake was not significant difference. However, it should be noted that during the first 2 weeks after ovariectomy, the E_2 rats was likely to consume less food than the Ovx rats. Later, in part 3, the rats were first ovariectomized for 3 weeks to warrant the depletion of circulating estrogen and then the administration of estrogen or vehicle was continued for another 4 weeks. In this part, the results showed that the body weight of Ovx rats was increased during the last 4 weeks; while, the body weight of E_2 rats was decreased. During this time, the food intake between these two groups was not significant different. The results in term of body weight were consistent with previous studies (Wegorzewska et al., 2008; Kalandakanond-Thongsong et al., 2012). The possible reason was the modulation of eating behavior and/or body metabolism by estrogen. Musatov et al. (2007) suggested that the decrease in food intake and body weight by estrogen may occur via ERα activation as the ERα knock-out mice ate more than wild type mice. Further supported by the administration of ERα agonist but not ERβ agonist in the Ovx-rats, the injected rats had smaller meal size thus the total food intake was reduced (Santollo et al., 2007; Thammacharoen et al., 2009). Therefore, estrogen may act on ERα and lead to reduce food intake. Additionally,

estrogen may also affect body weight gain by modulated fat metabolism as demonstrated by Heine and co-workers (2000). They found that the visceral adiposity was increased in site specific ERα knock-out mice, while the daily food intake did not change. These results indicated that the increased in body weight in ERα knock-out mice may be due to change in energy metabolism rather than changes in food intake (Heine et al., 2000). Later, Zengin and co-workers (2012) demonstrated that the Ovx mice had lower oxygen consumption and energy expenditure and tended to reduce resting metabolic rate; they suggested that this effect was mediated via neuropeptide Y (NPY) as the reduction in metabolic rate was ablated in the NPY-knockout-Ovx mice. In the present study, the basal metabolic rate or the visceral fat measurement was not done; therefore, it could not rule out the definite role of estrogen on the energy expenditure. Further, the results in the part 3 suggested that E_2 may have more effect on metabolism as the reduction of body weight in E_2 treated rats was evident with no difference in food intake. It is thus likely that the increased in body weight of ovariectomized rat may be partially caused by both the lower basal metabolic rate and the higher food intake.

The effects of estrogen deprivation on uterine weight

The uterine weight was used as an indicator of estrogen depletion in this study. Moreover, the uterine weight is accepted as the gold standard for screening for estrogenic effects, known as the uterotrophic assay (Clode, 2006). In this study, the lower uterine weight in the Ovx rats confirmed the lower level of estrogen. In part 1 of the experiment where the rats were ovariectomized and received estrogen or vehicle for different periods of time; the data revealed that the uterine weight was started to decline as early as 1 week following ovariectomy and further declined toward the end of experimental part 1. In part 3 of the experiment, the Ovx rats were in fact depleted of estrogen for 7 weeks; while the E_2 rats were estrogen depleted for 3 weeks and later supplemented with estrogen for 4 weeks. In the E_2 rats, the uterine weight was higher than the Ovx rats, indicating the uterotrophic effect of estrogen. These results supported the successiveness of estrogen in reaching and activating its target organs.

The effects of time of estrogen deprivation on anxiety-like behavior using behavioral test, the elevated T-maze (ETM)

In the part 1, the effect of estrogen withdrawal was done in a timely manner by performed behavioral test at 1-4 weeks following ovariectomy. The results from the ETM demonstrated that there was a significant difference in anxiety levels only in the Ovx rats but not the estrogen replacement rats. This confirmed the anxiolytic effect of estrogen. In the Ovx rats, the latencies of the inhibitory avoidance trial 1 and 2 of the 21- and 28- day Ovx rats were longer than 7- and 14- day Ovx rats with a significant negative correlation between the time and the level of anxiety. These data indicated that the longer the estrogen withdrawal, the higher the anxiety level. It should be noted that in the 14- day Ovx, some animals started to show sign of anxiety (~30%) but were more consistent at day 21 to 28 (approximately 60 and 90%, respectively). This result indicated that estrogen deprivation as early as 21 days can induce anxiety in female rats.

A number of research studies have been done in order to elucidate the anxiolytic effect of estrogen; however, various findings including anxiolytic, anxiogenic and no effect have been reported. One possible factor affected the findings could be the length of estrogen deprivation following ovariectomy. In these previous studies, behavioral testing began at different times after ovariectomy, ranging from 2 – 24 weeks and time of estrogen replacement after ovariectomy were started varied from 1 day to a delay of 1 week (Galeeva and Tuohimaa, 2001; Morgan and Pfaff, 2001; 2002; Imwalle et al., 2005; Hiroi and Neumaier, 2006; Pandaranandaka et al., 2006; 2009; Walf et al., 2009; Kalandakanond-Thongsong et al., 2012). It was likely that the longer the estrogen deprivation, the more consistent anxiolytic effect of estrogen was reported (Frye and Walf, 2004; Pandaranandaka et al., 2006; 2009; Walf et al., 2009; Lagunas et al., 2010; Diz-Chaves et al., 2012; Kalandakanond-Thongsong et al., 2012). On the other hand, when the length of deprivation was briefed, the estrogen administration has been reported to either produce no effect (Galeeva and Tuohimaa, 2001; Imwalle et al., 2005) or anxiogenic effects (Morgan and Pfaff, 2001; 2002). These previous reports was then supported by the current study as it was shown here that the anxiety can be achieved more uniformity after 3-week of ovariectomy.

Surprisingly, while the uterine weight was started to decline as early as 1 week following ovariectomy and further decline toward the end of experiment; the behavioral changes in term of anxiety were under detected until 3-4 weeks after ovariectomy. This discrepancy was rather interesting and may be related to the alteration of serotonergic and/or GABAergic system.

The effects of time of estrogen deprivation on serotonergic activity in brain areas associated with anxiety

The neurotransmitter serotonin has been implicated in the regulation of anxiety which can be modulated by estrogen (Donner and Handa, 2009; Hiroi and Neumaier, 2009; Pandaranandaka et al., 2009). Therefore, the serotonin levels in brain regions associated to the anxiety-like behavior, i.e. midbrain, amygdala, frontal cortex, hippocampus, nucleus accumbens, septum and anterior hypothalamus were also determined using HPLC-EC. The HPLC data revealed the consistency increased of 5-HT and 5-HIAA levels in various areas of the brain at day 28 in both Ovx and E_2 groups. It is thus likely that the 5-HT and 5-HIAA levels were increased with age. The age dependent of 5-HT level had been reported by previous studies; the 5-HT level has been reported to be low during development and it was elevated when adults (Giulian et al., 1973; Borue et al., 2007; Olivier et al., 2011). Additionally, Olivier et al. (2007) also reported that the 5-HIAA in the hippocampus and dorsal raphe of the rat at age 16 months was increased when compared to the rat at age of 5 months. Further, Shim et al. (2012) reported that the 5-HT levels in the inferior colliculus and medial geniculate body of the rat at the age of 24 months were higher than those at the age of 2 weeks. Since the comparison in 5-HT and 5-HIAA levels were done between groups at different age; it is not clear whether the difference in the 5-HT and 5-HIAA levels was due to the rats' age or estrogen condition.

Although the levels of 5-HT and 5-HIAA were increased by age in both Ovx and E_2 groups, the 5-HT turnover rates were differed. In the E_2 groups, the 5-HT turnover rate was rather consistent in most examined brain areas; on the other hand, the 5-HT ratios of the Ovx groups were more fluctuated. The pronounced effects were seen in the midbrain, the major clustering site of serotonergic neuron, and the

amygdala, the innervations of serotonergic neuron. In the midbrain, the levels of 5-HT and 5-HIAA were dramatically decreased at day 21 with the turnover rate being highest especially compared to at day 28. In the amygdala, the 5-HT and 5-HIAA levels were increased at day 14, decreased at day 21 and then increased again at day 28; this pattern was somewhat different from those supplemented with estrogen. Considering the turnover rate of 5-HT, the turnover rate was decreased in parallel with time of estrogen deprivation, suggesting that serotonergic activity was decreased.

When the behavioral data were brought together with 5-HT levels, at 3 weeks after ovariectomy at which the fluctuation of the 5-HT activity in the midbrain and amygdala were most obvious, it was the same time that the anxiety behavior was uniformly occurred. These data therefore suggested the interconnection of the 5-HT and the anxiety behavior. The disturbances of serotonergic activity as indicated by the 5-HIAA/5-HT ratio had been shown to be related to the increased in anxiety level (Pandaranandaka et al., 2009; Guimaraes et al., 2010). Then, the increase 5-HIAA/5-HT ratio in the midbrain and the decrease 5-HIAA/5-HT ratio in the amygdala at day 21 after ovariectomy may be one factor initiated the anxiety behavior as measured by the ETM test.

The effects of time of estrogen deprivation on gene expression of $GABA_A$ receptor subunits in the midbrain and the amygdala

The involvement of GABAergic system in regulating serotonergic systems is well established (Gervasoni et al., 2000; Tao and Auerbach, 2000; Castilho et al., 2002). Local applications of $GABA_A$ receptor agonist, muscimol into the midbrain diminish serotonergic activity in the midbrain and forebrain, which could be blocked by $GABA_A$ receptor antagonist, bicuculline (Tao and Auerbach, 1994; Tao et al., 1996; Li et al., 2005). The alteration of $GABA_A$ receptor subunits following ovariectomy in midbrain and amygdala was thus investigated in this study, as the dynamic changes of $GABA_A$ receptor expression during fluctuation of ovarian hormone have been previously reported (Smith et al., 1998a, b; Gulinello et al., 2003; Smith et al., 2006; Griffiths and Lovick, 2005; Lovick et al., 2005; Byrnes et al., 2007; Maguire and Mody, 2007). Based on the findings that α2 and α3 subunits were

responsible for the anxiolytic effects of benzodiazepine and α4 subunit was insensitive to benzodiazepine, these subunits were selected to test a relevance of estrogen deprivation on gene expression. Utilizing real-time PCR technique, the different pattern of $GABA_A$ receptor subunit expression in midbrain and amygdala was demonstrated. After estrogen withdrawal, the α2-, α3- and α4 $GABA_A$ receptor subunit gene expressions in the midbrain were higher in the Ovx than the E_2 groups especially the α2- and α3- $GABA_A$ receptor subunits. In the E_2 groups, the expression levels were rather stable. Contrarily, for the Ovx groups, the α2 receptor subunit was higher at day 28 while the α3- and α4- receptor subunits were markedly up-regulated at day 21 and started to down-regulate at day 28. The up-regulation of α4-, β1- and δ $GABA_A$ receptor in the midbrain during low estrogen levels had been demonstrated earlier by Lovick et al. (2005). In the amygdala, there was no statistically different expression in the α2-, α3- and α4 receptor subunits, and this was in agreement to the study by Noriega et al. (2010). However, it should be noted that the expressions of α2-, α3- and α4 receptor subunits were uniformly decreased at day 21 and likely to be continually decreased in the Ovx groups. The different expression response of $GABA_A$ receptor subunits between the midbrain and the amygdala following ovariectomy was not surprised. Noriega and colleagues (2010) demonstrated that the up- or down- regulation of $GABA_A$ receptor subunits were depended on the brain areas as the levels of α2-, α3- and α4 receptor subunits in the amygdala were unchanged while the α1- and α4 receptor subunits in the hippocampus were lowered in the estrogen treated ovariectomized rhesus monkey. These dynamic gene expressions of the $GABA_A$ receptor subunits according to estrogen level may be involved in the neuronal adaptation to maintain their functions.

At day 21 after ovariectomy during which the levels of α3 and α4 were up-regulated and the α2 was down-regulated in the midbrain. It had been shown that the up-regulation of the $GABA_A$ receptor α4 subunit was insensitive to benzodiazepine (Wafford et al., 1996) and associated with increased anxiety (Smith et al., 1998b). The increased anxiety could be due to the fact that the EC_{50} for GABA of the α4β1δ $GABA_A$ receptor was nearly 15 times lower than α1β2γ2 $GABA_A$ receptor as demonstrated by the recombinant study (Lovick et al., 2005). It is thus likely that the

dramatically increase of α4 subunit in the midbrain at day 21 (28 fold higher than the E_2 counterpart) in conjunction with the decreased expressions of α2, α3 and α4 in the amygdala may be partially responsible for the anxiety levels as measured by the ETM test.

Further, the current study also demonstrated that the $GABA_A$ receptor α2 subunit in the midbrain of Ovx rat was up-regulated at day 28 after ovariectomy. This subunit was known to mediate anxiolytic effect (Low et al., 2000) and the up-regulation of this subunit was found to be associated with steroid hormone (Pierson et al., 2005; Byrnes et al., 2007). In *vitro* and in *vivo* studies demonstrated that estrogen increased the α2 subunit expression in the brain (Herbison and Fenelon, 1995; Pierson et al., 2005; Byrnes et al., 2007) and was not depended on the GABA levels (Fenelon and Herbison, 2000). Therefore, one plausible explanation for the up-regulation of the $GABA_A$ receptor α2 subunit after ovariectomy in this study was the involvement of neuronal adaptation in order to maintain their functions following estrogen deprivation. However, behavioral data revealed that the ovarictomized rats were more anxiety at day 28 after ovariectomy suggesting that the inadaptation of the neuron after long-term estrogen deprivation.

Altogether, the conclusion that could be drawn from this part of the experiment was that after 3 week-ovariectomy, the circulating of estrogen was decreased as evident by the reduction in uterine weight of the Ovx rats. At this point, the anxious behavior was uniformity revealed concomitantly with the dramatical changes in serotonergic activity and $GABA_A$ receptor subunit mRNA expressions especially in the midbrain and the amygdala, the anxiety regulating area.

From above, it was thus interesting to determine whether the changes in gene expression of $GABA_A$ receptor subunits could lead to functional changes and/or modulate the serotonergic activity because the co-localization of GABA receptor on the serotonergic neuron had been demonstrated (Wirtshafter and Sheppard, 2001).

The effect of benzodiazepine agonist on the anxiety-like behaviors and locomotor activity

The effect of estrogen deprivation on the $GABA_A$ receptor function in relation to anxiety behavior was investigated in part 2 and 3. In part 2, the ovariectomized rats with or without estrogen supplementation for 3 weeks were used; the $GABA_A$ receptor function was determined by injecting diazepam, the benzodiazepine agonist, at the dosages of 0, 0.25, 0.5 and 1 mg/kg. In part 3, the 3-week-ovariectomized rats were subsequently supplemented with or without estrogen for 4 weeks before testing the $GABA_A$ receptor function by injecting with diazepam at the dosage of 0.25 mg/kg. The behavioral data from parts 2 and 3 suggested that there was a different response to benzodiazepine agonist, diazepam between Ovx and estrogen replacement rats. In the Ovx rats, the anxiolytic effect of diazepam was seen when the diazepam was given at the dose of 0.25 mg/kg as demonstrated by the decreased latencies of the inhibitory avoidance trial 2 compared to the vehicle treated rat. At higher doses (0.5-1.0 mg/kg), the inhibitory avoidance trial 2 latencies were not different from the vehicle group; in this case, it may be interpreted as the rats were more anxiety or the rats were sedated. The latter may be more reasonable as determined from the locomotor activity in the open field apparatus. It was clearly shown that the rats were less active as the numbers of line crossed was lower in the higher doses with significant effect at the dose of 1.0 mg/kg, suggesting the sedative effect of diazepam in these rats. Accordingly, the increase in escape latency of the ETM in the Ovx rats treated diazepam at the dose of 1 mg/kg was rather due to sedative effect rather than the anxiolytic effect in term of PD. In the E_2 group, there was no significant difference in any behavioral parameters observed from the ETM; however, the latencies of inhibitory avoidance trial 2 were likely to be lowered when diazepam was given at the doses of 0.25-0.5 mg/kg and higher at the dose of 1 mg/kg. Similarly to the Ovx rats, diazepam at the dose of 1 mg/kg affected the locomotor activity; thus, the effect seen in the ETM could interpret as the sedative effect of diazepam. The insignificant effect of diazepam in lowering anxiety in the E_2 rats may be that the anxiolytic effect of estrogen was maximized and could not further reduce by diazepam administration. Nevertheless, the behavioral data of the inhibitory

avoidance in the ETM of the Ovx and E_2 rats indicated that the responsiveness of $GABA_A$ receptor was indeed different.

Previous studies have shown that the $GABA_A$ receptor binding sites and subunits can be modulated by ovariectomy. For instance, the $GABA_A$ receptor subunits were up-regulated in several brain regions after ovariectomized for 4 weeks (Juptner et al., 1991). Further, Bosse and Di Paolo (1995) reported that the GABA-benzodiazepine binding site in substantia nigra was increased following 2 week-ovariectomy and progressively increased to 40 % at 3 months after ovariectomy. Later in 2006, Picazo and co-workers revealed that 12 weeks Ovx rats were more respond to benzodiazepine agonist than 3 weeks Ovx rats. Similarly, in intact female mice, female mice were more responsive to the anxiolytic effects of diazepam during estrus or diestrus phase, with no effect during late diestrus, proestrus or metestrus phase (Carey et al., 1992; Reddy and Kulkarni, 1999). These findings were in agreement with the current study parts 2 and 3; in that, the lower dosage of diazepam was able to induce sedative effect in the Ovx rats compared to the estrogen supplemented rats (0.5 vs. 1.0 mg/kg) indicated the higher responsiveness in the Ovx rats.

The increased responsiveness of $GABA_A$ receptor to its agonist could be due to various reasons. One possible explanation was the reduction of its ligand, GABA in the brain after ovariectomy (Nakamura et al., 2005). It was suggested that estrogen play a role in the regulation of glutamic acid decarboxylase, the rate limiting enzyme for GABA synthesis (Curran-Rauhut, and Petersen, 2002; Nakamura et al., 2005). Generally, glutamic acid decarboxylase consists of two isoforms, glutamic acid decarboxylase 65 and glutamic acid decarboxylase 67 (Soghomonian and Martin, 1998). Nakamura and co-workers (2005) revealed that the number of glutamic acid decarboxylase 65 in the hippocampus was down-regulation after ovariectomy for 10 days and estrogen can reverse this effect. This action was suggested to be mediated via ERβ as the ERβ agonist–treated male mice had higher expression of glutamic acid decarboxylase in cortex and hippocampus (Tan et al., 2012). These finding indicated that long-term estrogen deprivation induces a decrease in GABA levels in the brain and may lead to up-regulation of $GABA_A$ receptor expression in the brain. Therefore, the increased responsiveness of $GABA_A$ receptor to its agonist to benzodiazepine

agonist in Ovx groups as seen in the current study may be due to the fact that long-term ovariectomy (3 and 7 weeks in the study parts 2 and 3, respectively) was long enough to induce a decrease in glutamic acid decarboxylase expression resulting in lower GABA levels in the brain and lead to up-regulation of the $GABA_A$ receptor. This explanation was further supported by the results from part 1, in that the expression of $GABA_A$ α3 and α4 receptor subunit mRNA in the midbrain were dramatically up-regulation at 3 weeks following ovariectomy. This dynamic expression of the $GABA_A$ receptor subunit may be involved in the increase sensitivity to benzodiazepine agonist as seen in study part 2 and 3. Conclusively, the current study indicated that long-term ovariectomy can alter the $GABA_A$ receptor subunit expression and consequently alter their functions in the brain area regulating anxiety behavior. This alteration may be partially accounted for the etiology of anxiety. Further, the behavioral data also indicated that estrogen was not only able to prevent anxious behavior but it was also able to reduce anxiety in anxious rats. This effect of estrogen was proven by the results from part 2 and 3; in part 2, the estrogen treated rats had lower level of anxiety compared to the Ovx rats suggesting the preventive role of estrogen. In part 3, the rats were first induced to be anxious (based on the time dependent study of part 1) and then the anxiolytic effect of estrogen was evident after 4-week supplementation, suggesting the role of estrogen in treating anxiety.

The effect of benzodiazepine agonist on serotonergic activity in brain areas associated with anxiety

The results from the behavioral test revealed that the $GABA_A$ receptor sensitivity was altered after ovariectomy. A number of studies demonstrated the co-localization of GABA receptor on the serotonergic neuron (Wirtshafter and Sheppard, 2001) and the administration of $GABA_A$ receptor agonist into midbrain could reduce the levels of 5-HT in the midbrain and frontal cortex (Tao et al., 1996; Millan et al., 2001; Tao and Auerbach, 2003). In this study, the serotonergic activity in brain areas associated with anxiety was thus determined by measuring the 5-HT and 5-HIAA levels after the administration of benzodiazepine agonist. For both parts 2 and 3, similar results were demonstrated. Surprisingly, in the Ovx groups, there was no difference in the levels of 5-HT and 5-HIAA or the ratio of 5-HIAA/5-HT following

diazepam in all examined brain areas; on the other hand, the differences were demonstrated in the E_2 groups. Despite the difference, the changing in the levels of 5-HT and 5-HIAA or the ratio of 5-HIAA/5-HT in the E_2 groups was not corresponded to the dosages of diazepam; for instance, the 5-HT levels in the frontal cortex was significantly increased following diazepam injection at the doses of 0.25 and 1.0 mg/kg but not 0.5 mg/kg. Intriguingly, these data were not in accordance with the behavioral data; for example, in the Ovx group, the diazepam at the dose of 0.25 mg/kg altered the behavioral responses but not the 5-HT levels in all examined brain areas.

Previous studies reported that GABA has inhibitory effect on 5-HT release in the midbrain and frontal cortex (Tao and Auerbach, 2000; Millan et al., 2001). The local infusion of benzodiazepine agonist or $GABA_A$ receptor agonist into midbrain reduced the 5-HT levels in the midbrain and frontal cortex (Tao et al., 1996; Millan et al., 2001; Tao and Auerbach, 2003). On the other hand, infusion of $GABA_A$ receptor antagonist, bicuculline into the midbrain of awake and active rats increased the 5-HT level in the brain (Millan et al., 2001; Tao et al., 1996; Tao and Auerbach, 2003). Then, they suggested that GABAergic neurons in the dorsal raphe nucleus have a tonic inhibitory effect on serotonergic neuron. If this was the case, the level of 5-HT was then expected to be lower following diazepam administration. However, in the present study, the low dose benzodiazepine agonist had no effect on 5-HT discharge; whereas, the high dose tended to increase 5-HT levels. Before further discussion, it should be aware that the route of administration had to be taken into account as it could produce different effects. In most previous studies, the administrations were done directly into the specific brain area; thus, the affected neuron was relatively confined resulting in specific responses. On the contrary, the administration in this study was in fact systemic (subcutaneous injection); thus, the effect was indeed unrestricted and depended upon receptor expression. This is the limitation of the current work and makes it rather difficult to interpret.

The first notation that should be pointed out was that the 5-HT levels were increased especially in the midbrain, frontal cortex and hippocampus of the E_2 treated rats after diazepam administration. The increase in the 5-HT levels after diazepam administration may be involved in the modulation effect of diazepam on the

tryptophan (Pratt et al., 1985; Rastogi et al., 1977). Pratt et al. (1985) reported that acute administration of benzodiazepine agonist increased the levels of 5-HT, 5-HIAA and tryptophan. They suggested that the increase of 5-HT levels may be the results of elevate concentration of tryptophan, the substrate for 5-HT synthesis. Supported by Hockel et al. (1979), they reported the increased level of L [G-3] tryptophan in the brain after benzodiazepine agonist infusion which was occurred within 0.5 and 2.0 hr. These data supported that diazepam increased 5-HT levels by increased uptake of tryptophan into the brain. The increased in the tryptophan uptake was probably due to the increased in free serum tryptophan availability since benzodiazepine could displace more than 40% of the bound tryptophan from serum albumin (Muller and Wollert, 1975). Therefore, the increased 5-HT levels after administration with benzodiazepine agonist in the present study may be the consequence of the elevated serum free tryptophan by mean of diazepam displacement, leading to the increased brain uptake and more substrate available for the 5-HT producing enzyme, tryptophan hydroxylase and finally the levels of 5-HT in the brain were thus increased. Interestingly, this explanation was not applicable to the Ovx rats as in these rats the level of 5-HT was not altered after diazepam administration.

The second notion was the increase in the 5-HT levels of E_2 rats compared to the Ovx rats as seen in part 2. This data was consistent to previous reports, it was demonstrated that E_2 has many effect on the serotonergic system in the brain (Rubinow et al., 1998; Robichaud and Debonnel., 2005). Donner and Handa (2009) demonstrated that ERβ mediated estrogenic effect on the elevation of the tryptophan hydroxylase enzyme in the midbrain. In addition, estrogen also decreased the $5\text{-}HT_{1B}$ autoreceptor in the dorsal raphe nucleus (Hiroi and Neumaier, 2009). Therefore, the elevation of 5-HT levels in E_2 treated rats in the present study may be results of estrogen decreased $5\text{-}HT_{1B}$ autoreceptor and/or increased tryptophan hydroxylase enzyme in the midbrain, thereby increasing the ability for 5-HT synthesis and release in different brain areas associated with anxiety.

Although, the 5-HT levels in the Ovx rats were not changed after diazepam administration. However, the alteration of the $GABA_A$ receptor sensitivity after ovariectomy may be not only limited to the serotonergic system but also other neurotransmitter systems. For example, the modulation of norepinephrinergic and

dopaminergic systems by $GABA_A$ receptor had also been reported (Shekhar et al., 2002; Yee et al., 2010). Therefore, further studies are required to determine the roles of other systems in association with anxiety. Moreover, in order to clarify the role of $GABA_A$ receptor on the serotonergic system, the site-specific injection should be considerate.

CHAPTER VI
CONCLUSION

The overall objective of the present study was to investigate the effects of estrogen deprivation on anxiety-like behaviors, $GABA_A$ receptor subunit gene expression and function, and serotonergic activity in brain area associated with anxiety utilizing ovariectomized rat as a model. Firstly, the time required to induce anxiety was studied along with the changes in $GABA_A$ receptor subunit gene expression and serotonergic activity in brain area associated with anxiety. The rats were ovariectomized and later tested for anxiety with elevated T-maze; the brains were then collected for the measurement of $GABA_A$ receptor subunit gene expression and serotonin levels with real time PCR and HPLC techniques, respectively. Secondly, the $GABA_A$ receptor sensitivity was tested by injecting different dosages of benzodiazepine agonist, diazepam; concurrently, the $GABA_A$ alterations affect serotonergic activity was also investigated. The first and the second would provide the information on the roles of estrogen in causing and preventing anxiety. Finally, the role of estrogen in treating anxiety was studied. In this part, anxiety was first induced by ovariectomized and later supplemented with estrogen, the behavioral data along with the changes in $GABA_A$ receptor sensitivity and serotonin were investigated.

The conclusions that can be drawn from this dissertation are as follows.

1. Anxiety could be uniformly induced in female rats after ovariectomized for at least 3 weeks. The longer the lacking of estrogen, the higher the anxiety was demonstrated.
2. The alterations of $GABA_A$ receptor subunit gene expressions i.e. $\alpha 2$, $\alpha 3$ and $\alpha 4$ were found esp. in the midbrain of the estrogen-deprived rat. These alterations were likely to affect $GABA_A$ receptor sensitivity as these rats showed higher responsiveness to benzodiazepine agonist compared to estrogen supplemented ovarictomized rats. The changes in $GABA_A$ receptor subunit gene expressions and sensitivity were found in relation to the changes in behavior.

3. The serotonergic activity in the brain areas associated with anxiety as determined from serotonin, its metabolite and the ratio of its metabolite and serotonin indicated that there were age dependent as the levels of serotonin and its metabolite were increased as the rat aged. Despite the age effect, the serotonin turnover rates (the ratio of its metabolite and serotonin) in the estrogen supplemented rats were relatively stable; while it was more fluctuated in the estrogen deprived rats.

   From all above, the preventive effect of estrogen was therefore supported through the behavioral data, the alterations of $GABA_A$ receptor subunit and function, and the serotonergic activity.
4. Following the induction of anxiety by ovariectomy, subsequent estrogen supplementation could reduce anxiety as seen in behavioral data from the last part of the experiment. The reduction in anxiety was probably due to the regulative effect of estrogen on GABAergic and/or serotonergic system as demonstrated by the different in benzodiazepine agonist responsive between ovariectomized rats with or without estrogen supplementation. However, due to the limitation of this study, it could not rule out whether the alteration of $GABA_A$ receptor function after ovariectomy affected the serotonergic activity in brain areas associated with anxiety.

Conclusively, this dissertation provided the first evidence of the time of estrogen deprivation on anxiety levels; the more understanding mechanism in relevant to the timing of estrogen decline and the molecular mechanism of estrogen on the regulation of GABAergic system, which has important role in modulating emotion the brain was established.

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