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.