CHAPTER V
DISCUSSION

Several basic and clinical studies provided evidences for the complex effects of estrogen on the regulation of anxiety. In normal cycling women, transient high estrogen levels during the preovulatory surge and low levels of estrogen during the premenstrual phase were reported to be the reasons of anxiety symptoms (Arpels, 1996; Sigmon *et al.*, 1996). In addition, low estrogen levels during postmenopausal women showed anxiety symptoms which being alleviated by chronic ERT (Halbreich, 1997; Sherwin, 1998). These findings demonstrated possible differential effects of endogenous (acute) in menstrual cycle and exogenous (chronic) estrogen given in ERT. In experimental animals, similar inconsistent data have been found. Female rats during proestrous phase with the higher levels of endogenous estrogen than other phases exhibited anxiolytic-like behaviors when tested with the EPM (Mora *et al.*, 1996; Frye *et al.*, 2000; Marcondes *et al.*, 2001). In contrast, Nomikos and Spyraki (1988), utilizing the same behavioral model, showed that Pro rats were not significantly different in anxiety parameters. Chronic estrogen treatment in Ovx rats using the EPM, also manifested an anxiolytic property (Frye and Walf, 2004; Koss *et al.*, 2004; Pandaranandaka *et al.*, 2006). These unclear data regarding endogenous and exogenous estrogen on anxiety might be related to the animal model of anxiety to specify the different types of anxiety. We currently know that anxiety has been classified into different subtypes; e.g. PD, phobia, and GAD which reflected by different behavioral models of anxiety.

In 1993, Graeff and co-workers (1993) developed an animal model of anxiety to separate the different types of anxiety, namely the ETM. The advantage of this behavioral model is that it can evaluate two types of anxiety in the same animal, i.e. learned (or conditioned) anxiety, represented by inhibitory avoidance behavior, and innate (or unconditioned) fear, represented by one-way escape (Graeff *et al.*, 1993; Viana *et al.*, 1994; Zangrossi and Graeff, 1997) which is based on the hypothesis of the dual roles of 5-HT in controlling anxiety (Deakin and Graeff, 1991; Graeff, 1991; Graeff *et al.*, 1997). Moreover, pharmacological validation of this model has shown that this task was impaired by treatment with diazepam, ipsapirone, buspirone, ritanserin and chronic imipramine (Graeff *et al.*, 1998; Viana *et al.*, 1994),

treatments that were effective in GAD. Thus, the learned nature and its pharmacological sensitivity suggest that this behavior is related to GAD (Deakin and Graeff, 1991; Graeff *et al.*, 1998; Zangrossi and Graeff, 1997). On the other hand, one-way escape was increased by chronic treatment with mipramine and fluoxetine (Graeff *et al.*, 1998; Solyom, 1994), treatments that were effective on PD. Thus, based on the assumption that innate fear is related to PD (Deakin and Graeff, 1991) and on the pharmacological sensitivity of PD, one-way escape is supposed to represent panic anxiety (Deakin and Graeff, 1991; Graeff *et al.*, 1998; Zangrossi and Graeff, 1997).

Presently, it is well known that estrogen appeared to have closely linked to the serotonergic system (Shughrue *et al.*, 1997). It is therefore interesting to attempt to use the ETM to investigate the differential effects of endogenous (acute) and exogenous (chronic) estrogen on anxiety-like behaviors in female rats. Present study was divided into two parts. The first part studied the anxiety-liked behaviors of female rats. The second part was focused to investigate the effects of estrogen on serotonergic systems at the brain areas involving anxiety.

The effects of ovariectomy on body weight, food intake and uterine weight

After ovariectomy for 4 weeks, the deprivation of ovarian sex hormones in Ovx rats was confirmed by the reduction in their UW compared with Ovx+E_2 and Pro rats. Although, the plasma estrogen levels were not measured in this study to confirm the deprivation or the successive replacement of estrogen, there were a number of reports showing that higher levels of plasma estrogen may not reflect brain estrogen levels (Almeida *et al.*, 2005; Hsiao *et al.*, 2004). Further, Morgan and Pfaff (2002) did not find estrogen's effects to be dose-dependent since the levels of plasma estrogen might not correlate with anxiety or depression. This suggests that UW may be a better indicator of estrogen reaching its target organ and performing the associated physiological functions. However, it should be reminded that not only estrogen but also progesterone that regulate many physiological processes in the uterus at molecular, cellular, and organ levels (Iruela-Arispe *et al.*, 1999; Rogers *et al.*, 1998). While estrogen plays physiologically significant role in stimulating the uterine proliferation and differentiation of the uterus (Mellor and Thomas, 1995), progesterone activates differentiation by inducing transformation of the endometrium

to the secretory type (Buchanan *et al.*, 1999) causing the accumulation of secretion in uterus. In this study, the supplementation of estrogen to Ovx rats prevented the decrease in the UW of the animals; therefore, supporting the significant role of estrogen in maintaining uterine growth. Although, the UW of Ovx+E_2 was not as high as of Pro rats, it is possible that the Pro rats have high levels of both estrogen and progesterone. Further, from our observation, at the time of uterine sampling, it should be noted that the uterine of Pro group was filled with fluid. Additionally, we also observed the vaginal smear of Ovx and Ovx+E_2 rats (data not shown), in Ovx rats, the vaginal smear showed an atrophic pattern consisting of leukocytes, mucus and scarce nucleated epithelial cells while Ovx+E_2 rats consisted mainly of exfoliated cornified cells. These observations might confirm the completeness of ovariectomy and effectiveness of estrogen administration. Moreover, the deprivation of ovarian sex hormones was further confirmed by the changes in BW. The results showed the significant increase in BW and DWG after ovariectomy in Ovx rats as compared to Ovx+E_2 and Pro rats. This finding is in agreement with previous reports from other laboratories (Gray *et al.*, 1993; Shimomura *et al.*, 1990; Thomas *et al.*, 1986). It has been shown that estrogen-sensitive neurons are present in the ventromedial arcuate region and the anterior hypothalamus-preoptic region in which estrogen can modulate the eating habit (Dagnault and Richard, 1997; Palmer and Gray, 1986). These may involve in an increase in food consumption representing by increasing in DWG after ovarian sex hormone deprivation and then lead to an increase in BW of the Ovx rats, as present in this study.

From these results, we conclude that 4 weeks following ovariectomy induced estrogen deprivation in Ovx rats and replacement with estrogen at 10 μg/kg can present the lack of estrogen in Ovx rats at shown by the changes in UW, BW, DWG, and vaginal smear in these animals.

The differential effects of endogenous (acute) and exogenous (chronic) estrogen on anxiety-like behaviors using ETM test

In present study, the effects of chronic treatment of estrogen on the Ovx rats and the natural high levels of estrogen in Pro rats involving anxiety levels were investigated with the ETM, an animal model of innate (panic) and learned (generalized) anxiety. It demonstrated that Ovx+E_2 rats have lower levels of GAD than others as demonstrated by the shortened inhibitory avoidance latency; while the Pro rats have lower level of PD than others as shown by the increase in escape latency. Additionally, the data from the open field revealed that only the Ovx+E_2 group showed the anxiolytic-like behavior by increasing the time spent in the inner zone. Since the locomotor activity as indicated by the total numbers of crosses in open field arena, were not different among these animals, we conclude that the changes in anxiety parameters obtained in the ETM were not due to locomotor alteration. Moreover, it was suggested that the memory can alter the animal performance in the ETM, the impaired inhibitory avoidance of the Ovx+E_2 group may due to changes in learning and memory processes rather than changes in anxiety processes. The deficit in memory of Ovx+E_2 rats can be ruled out because they showed significantly increase latency along inhibitory avoidance trials, suggesting that acquisition and short-term memory were intact. Additionally, a similar pattern of ETM data was also presented in the open field data. One possible explanation of the Pro group did not show the anxiolytic-like behaviors demonstrated in the open field could be that this test was effective only to differential anxiety in term of GAD. Since, it has been that the drugs for treatment of GAD especially classical BDZs such as chlordiazepoxide and diazepam showing an anxiolytic effect in the open field test (Menard and Treit, 1999).

The results of Ovx+E_2 by lowering levels of GAD presenting in ETM test supported prior human studies in that the chronic treatment (2-3 weeks) of estrogen diminishes anxiety and/or irritability in peri- and post-menopausal women (Schmidt *et al.*, 2000; Sherwin and Gelfand, 1989; Thomson and Oswald, 1977). In addition, the results of Pro rats showed high levels of GAD also supported the human clinical report in that during the preovulatory surge, anxiety is the most common symptom which is strongly related to an excess of estrogen and a deficiency of progesterone in this phase. Moreover, the results of this present study also supported

the animal models. Previous studies have shown similar anxiolytic-like action following estrogen treatment in Ovx rats in the EPM (Diaz-Veliz *et al.*, 1997; Koss *et al.*, 2004; Nomikos and Spyraki, 1988; Pandaranandaka *et al.*, 2006;), in the open field (Blizard *et al.*, 1975; McCarthy *et al.*, 1995), and in a conflict test of anxiety (Rodriguez-Sierra *et al.*, 1984). However, the results of proestrus in anxiety still inconclusive in previous studies, some studies in rats have indicated that the proestrus female rats have lower levels of anxiety than the other phases of the cycle tested with EPM (Marcondes *et al.*, 2001; Mora *et al.*, 1996). In contrast, Nomikos and Spyraki (1988) showed no significant difference between any estrous phases. A plausible explanation for this inconsistent data of proestrus on anxiety levels measure by EPM may be that the EPM is a mixed model, in the sense that multiple defense reactions are displayed while the rats freely explored the apparatus.

For the conflict results between human and animal models about the natural high levels of estrogen in estrus cycle involving in anxiety, this phase in human stimulate anxiety but in animal models some data showed low levels of anxiety or no effect. The present results may clarify this conflict that the Pro rats have high levels of anxiety in the sense of GAD but low levels of anxiety in the sense of PD. For the possible explanation of the different effects of chronic estrogen treatment in Ovx rats and the natural high levels of estrogen in proestrous rats, it could be related to the genomic and nongenomic actions estrogen and the still lacking of progesterone in Ovx+E_2 group. Conclusively, regardless of different roles of estrogen (acute or chronic) affecting various forms of anxiety, we confirmed the anxiolytic like effect of estrogen. Since lacking estrogen for 4 weeks induced both GAD and PD in Ovx animals when tested with ETM which could alleviated by estrogen.

The differential effects of endogenous (acute) and exogenous (chronic) estrogen on serotonergic system

To further clarifying of the mechanisms of estrogen in controlling anxiety levels in females, we have studied the actions of this hormone on aspects of 5-HT neural function. The serotonergic system was examined in the brain areas involving anxiety of Ovx+E_2, Ovx and Pro rats immediately after the behavioral tests. The results of HPLC analysis showed that 5-HT levels of Ovx+E_2 rats tended to reduce in various brain regions. Moreover, the hippocampus and the nucleus accumbens of Ovx+E_2 rats also showed significantly increased in 5-HT turnover rate representing by 5-HIAA/5-HT ratio. However, it should be noted that the significant changes in turnover rate were likely due to the reduction of 5-HT levels rather than the increased in activity since the metabolites were not likely affected. This is consistent with results of Western blot analysis measuring TPH and SERT protein levels in which the Ovx+E_2 and the Pro rats have low levels of TPH protein while SERT protein levels were not affected as compared with estrogen deprivation rats. Finally, the results of treatment with 5-$HT_{2A/2C}$ antagonist showed that estrogen might involve 5-HT_{2A} and 5-HT_{2C} receptors function to control anxiety in both Ovx+E_2 and Pro rats, but the results have not yet been elucidated.

The relationship between estrogen and serotonergic system has been widely studied. It has been shown that estrogen may modulate this system by influencing synthesis, release, reuptake, or catabolism of the neurotransmitter (Lu *et al.,* 2003; McEwen, 2002; McQueen *et al.,* 1997). This effects of estrogen were probably mediated through the ER_β, as the increase in anxiety was evident in the ER_β knockout mice (Krezel *et al.,* 2001) while ER_β selective substance can decrease anxiety when given to Ovx rats (Walf and Frye, 2005). These indicated that the ER_β has a role on regulating anxiety. The immunochemical data has further confirmed the possible modulation of the serotonergic system through the ER_β, the ER_β mRNA and protein were found in raphe nuclei (Gundlah *et al.,* 2001), the originating pathway of serotonergic neurons. The ER_β expression was not limited to raphe nuclei but also extended to other brain areas including amygdala, hippocampus, and frontal cortex (Shughrue and Merchenthaler, 2000; 2001). A number of experiments have measured 5-HT metabolisms in brain following estrogen treatment in Ovx animals. Several

authors reported both an increase and a decrease in 5-HT levels, 5-HT turnover rate, and 5-HT reuptake after estrogen administration depended on areas of the brain examined and species of experimental animals. The low 5-HT levels of Ovx+E_2 rats shown in this study was consistent with previous study from our laboratory (Pandaranandaka *et al.*, 2006) and others (Cohen and Wise, 1988; Gereau *et al.*, 1993; Heikkinen *et al.*, 2002; Luine *et al.*, 1998); however it was inconsistent with some others (Cone *et al.*, 1981; Di Paolo *et al.*, 1983; King *et al.*, 1986; Morissette *et al.*, 1990; Renner *et al.*, 1986). Previous study from our laboratory (Pandaranandaka *et al.*, 2006) have reported the low 5-HT levels of Ovx+E_2 rats in frontal cortex, hippocampus, caudate putamen, nucleus accumbens, and substantia nigra as compared with Ovx rats. Many other studies also reported that levels of 5-HT were decreased in frontal cortex (Luine *et al.*, 1998), hippocampus (Heikkinen *et al.*, 2002), and discrete hypothalamic nuclei, particularly in the suprachiasmatic nucleus and ventromedial nucleus (Cohen and Wise, 1988; Gereau *et al.*, 1993; Jame *et al.*, 1989) of Ovx+E_2 rats. In contrast, other studies reported an increase in 5-HT levels and 5-HIAA levels in dorsal raphe (Cone *et al.*, 1981; Di Paolo *et al.*, 1983; Renner *et al.*, 1986) and in some nuclei of hypothalamus (King *et al.*, 1986; Morissette *et al.*, 1990). The effects of estrus cycle on the serotonergic system have also been reported. Felton and Auerbach (2004) recently demonstrated that during proestrus and estrus, baseline 5-HT levels were significantly higher compared to Ovx rats. Moreover, rats with equal emotional reactivity in proestrous phase diminished 5-HIAA and 5-HIAA/5-HT ratio in the hippocampus and 5-HIAA/5-HT ratio in the hypothalamus-preoptic area (Sfikakis *et al.*, 2002).

Serotonergic neurotransmission is governed by various regulatory proteins in both serotonergic neurons and their targets. Two pivotal control proteins are TPH, the rate limiting enzyme in 5-HT synthesis, and the SERT, which is blocked by the widely prescribed class of anxiolytic drug known as SSRIs. Changes that occur in either of these proteins could have widespread repercussions in serotonergic transmission and, in turn, changes anxiety-liked behaviors. This study demonstrated that TPH protein content is high in the midbrain of Ovx rats while both exogenous and endogenous estrogens have lower. If TPH protein predicts 5-HT synthesis, then lacking ovarian hormones could lead to high 5-HT synthesis and, in turn, increased vulnerability to GAD. However, information regarding the effect of estrogen on TPH

expression or activity is limited and species differences have emerged. TPH mRNA expression in the DRN has been shown to increase with estrogen treatment in macaques (Pecins-Thompson *et al.*, 1996) and mice (Gundlah *et al.*, 2005), but not in rats (Alves *et al.*, 1997). TPH protein levels have also been shown to increase with estrogen treatment in DRN of macaques (Bethea *et al.*, 2000) and guinea pigs (Lu *et al.*, 1999). Regulation of TPH protein levels in the midbrain raphe by estrogen in rats has not yet been reported. The mechanisms by which estrogen may modulate the serotonergic raphe neurons are still unclear. It is possible that estrogen affect TPH protein expression both directly (via regionally distinct expression of ERs) and indirectly (via afferents to serotonergic neurons from steroid-sensitive brain regions).

Another key regulatory process of serotonergic neurotransmission is an inactivation of neurotransmitter following its stimulated release. This inactivation process is mediated by membrane-integral, SERT protein. The SERT proteins are predominantly located presynaptically at serotonergic nerve terminals (Hoffman *et al.*, 1998). Such this transporter protein has accessed to the extracellular domains of neuronal synaptic clefts. Dysfunction of SERT-mediated uptake of 5-HT has been implicated in anxiety disorders (Siever *et al.*, 1991). In addition, this transporter is intimately involved with anxiolytic drugs. Thus, by altering the expression or activity of SERT, could consequently alter serotonergic neurotransmission. In macaques, short-term treatment (28 days) of spayed animals with estrogen led to decrease amounts of SERT mRNA as compared to controls (Pecins-Thompson *et al.*, 1998). However, longer period of treatments (5 months) with estrogen actually led to increase amounts of SERT mRNA (Pecins-Thompson *et al.*, 1998). Thus, estrogen treatment appeared to have a variable effect on gene expression of SERT depending on the duration of estrogen therapy. In rats, it was reported that estrogen increased SERT binding sites in amygdala, lateral septum, and hypothalamus and also increased SERT mRNA expression in the DRN of animals that were Ovx, immediately injected with estrogen and killed the following day (McQueen *et al.*, 1997). However, the present study could not reveal any change in SERT protein levels in all brain regions of all groups of rats. However, the nongenomic effect of estrogen in inhibiting SERT function might be occurred especially in Pro rats. From *in vitro* study by Chang and Chang (1999) indicated that estrogen was capable of inhibiting SERT via a nongenomic mechanism. They found that estrogen exhibited noncompetitive

inhibition of radiolabeled 5-HT ([^{3}H]5-HT) transport by binding to this transporter. Such inhibitory effects were observed within short time courses and unlikely to result from genomic effects normally ascribed to estrogen action. Nongenomic effects of estrogen have also been reported to regulate other ion channel and other receptor activities in sharing several functional commonalities (McEwen, 1991; Morley *et al.*, 1992).

The present results show that the mixed 5-$HT_{2A/2C}$ antagonist, ritanserin, only at the dosage of 3.0 mg/kg tended to affect the inhibitory avoidance task of ETM by reducing avoidance latency of Pro rats showing that this dosage of this drug could reduce GAD in these rats. In addition, in the same dosage of ritanserin, Ovx rats tended to be decreased anxiety by increasing time spent in inner zone and decreasing time spent in outer zone of open field test. Ritanserin binds to both 5-HT_{2A} and 5-HT_{2C} receptors with different affinity (Hoyer *et al.*, 1994). Previous study was shown that the 5-HT_{2C} agonists, mCPP and TFMPP, had an effect on inhibitory avoidance indicating that the stimulation of these receptors increases conditioned fear (Mora *et al.*, 1997). Furthermore, the 5-HT_{2C} antagonists, SB 200646A and SER 082 had an anxiolytic effect on inhibitory avoidance, indicating that 5-HT_{2C} receptors are involved conditioned fear (Mora *et al.*, 1997). Moreover, anxiolytic effects of SB 200646A have been reported in the rat social interaction test (Kennett *et al.*, 1994) as well as in the rat Geller-Seifter model and in another conflict test in the marmoset (Kennett *et al.*, 1995). The above evidences with many additional literatures; therefore, showed the favorable of a participation of the 5-HT_{2C} receptor in the regulation of conditioned fear. However, the role of the 5-HT_{2A} receptor in conditioned fear is far less clear than that of the 5-HT_{2C} receptor. The results of Mora *et al.* (1997) showed that the preferential 5-HT_{2A} receptor agonist, DOI, was ineffective on inhibitory avoidance in the range of doses used. While the selective 5-HT_{2A} antagonist, SR 46349B, had a neat anxiolytic effect on inhibitory avoidance, and the highly selective agent, RP 62203, was ineffective. Therefore, the present results of ritanserin, the mixed 5-$HT_{2A/2C}$ antagonist, at a high dose (3.0 mg/Kg) had a modest anxiolytic effect, this may be due to its interaction with 5-HT_{2C} receptors. Consideration of ritanserin, the effects of single drug administration on animal models are inconsistent, because anxiogenic, anxiolytic, and null effects have been described (Griebel, 1995).

There are reports about estrogen increasing the gene expression and/or binding potentials of 5-HT_{2A} receptor, which may be of relevance to interfere the effect of ritanserin finding in this study by changing the balance of 5-HT_{2A} and 5-HT_{2C} receptors. The activation ER_{β} resulted in upregulation of the 5-HT_{2A} receptor (Österlund *et al.*, 1999). In addition, increasing estrogen caused an increase in the density and binding of the $5HT_{2A}$ receptor (Kugaya *et al.*, 2003; Moses-Kolko *et al.*, 2001) consistent with increasing of $5HT_{2A}$ density in post-menstrual women (Biegon and Greuner, 1992). A human positron emission tomography scan study in postmenopausal women also demonstrated that binding of altanserin (a selective 5-HT_{2A} receptor radioligand) to the 5-HT_{2A} receptor was increased by 21% following treatment with transdermal estrogen and micronized progesterone (Moses *et al.*, 2000).

To date, the interaction between the serotonergic system and the regulation of anxiety has been recognized. With regard to the 5-HT hypothesis, activation of the serotonergic neuronal function induces the anxiety-liked action, whereas a reduction of serotonergic function brings about the anxiolytic effect (Clement and Chapouthier, 1998; Rex *et al.*, 1993; 1994). Deakin and Graeff (1991) proposed that an ascending 5-HT neural pathway, originating from the dorsal raphe nucleus and innervating forebrain regions, including the frontal cortex, hippocampus, and amygdala, could facilitate the generation of anxiety by releasing 5-HT. There is apparently evidence to support this hypothesis. Administration of a 5-HT-releasing agent or a 5-HT agonist was shown to induce anxiogenic actions in patients with PD, as well as in animal models (Charney *et al.*, 1987; Tao *et al.*, 2002; Targum, 1990). The selective 5-HT_{1A} receptor agonist, resulted in decreased 5-HT release and showed anxiolytic effects by markedly decreasing the concentration of 5-HT (Matos *et al.*, 1996). Additionally, Ge and co-workers (1997) demonstrated that aversive stimuli (following social interaction testing) increased the turnover rate of 5-HT and DA in all parts of the brain, particularly the mesolimbic system, which is closely linked to anxiety. Our data is somewhat consistent with these reports in that the Ovx+E_2 rats with lower levels of GAD had also low levels of TPH protein in midbrain, which may play an important role in the anxiolytic effect of this hormone by decreasing the synthetic capacity for 5-HT in this brain region and thereby reducing serotonergic

input to specific forebrain projection areas. Interestingly, in this study, we also found the positive correlation between active avoidance 2 latency time, GAD-liked behavior in ETM, and 5-HT levels in septum, hippocampus, and amygdala of Ovx rats with and without estrogen administration. It was meant that high 5-HT levels correlated with high GAD levels in Ovx rats. However, the role of naturally high estrogen levels controlling anxiety in Pro group is still unclear. The Pro rats had low levels of TPH protein at midbrain, but they tended to have high levels of 5-HT in various brain regions equally to Ovx rats. This different of Ovx+E_2 and Pro rats in serotonergic profile may contribute to the different of actions of estrogen in controlling anxiety through this neurotransmitter system. As previous mention about the nongenomic effect of estrogen inhibiting SERT function, short period of natural high levels of estrogen in Pro rats may decrease the SERT function in the brain areas of serotonergic projection, e.g. hippocampus, frontal cortex, and amygdala, inducing increase in 5-HT levels in synaptic area lead to activate GAD-liked behaviors and anti-PD, simultaneously. Further, we should keep in mind that the measurements of 5-HT and its metabolite with the homogenate brain tissue used in this study was actually a summation of changes, both intra- and extra-cellular, since they failed to capture the in vivo dynamics of the monoamines transmissions. Furthermore, the effect of estrogen was not limited to serotonergic system but included other monoaminergic systems (NE, DA) (Etgen and Karkanias, 1994; Thompson and Moss, 1994) and amino acid transmitters (GABA, Glutamate) (Herbison, 1997; Kia *et al.*, 2002) and these transmitters have also been correlated with anxiety (reviewed by Millan, 2003).

In conclusion, the present results suggest an anti-GAD in chronic estrogen treated Ovx rats and anti-PD in the natural high levels of estrogen in proestrus females. The differences in serotonergic response from endogenous and exogenous estrogen activation may have implications in elucidating the complex regulation of anxiety by estrogen.