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).

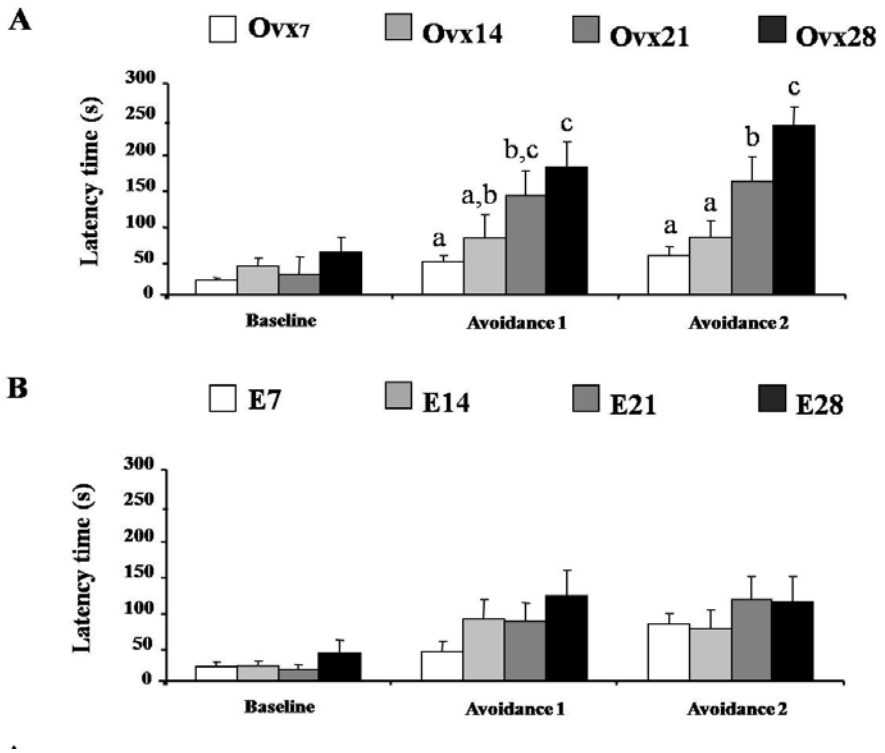

Figure 4-1 The effects of estrogen deprivation on anxiety-like behavior at each time point in (A) Ovx groups and (B) E_2 groups. Data present as mean $\pm$ SEM; ^{a,b,c} Different letters indicate statistical differences ($P<0.05$) among groups in the same trial. n = 10-12 for each subgroup.

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.

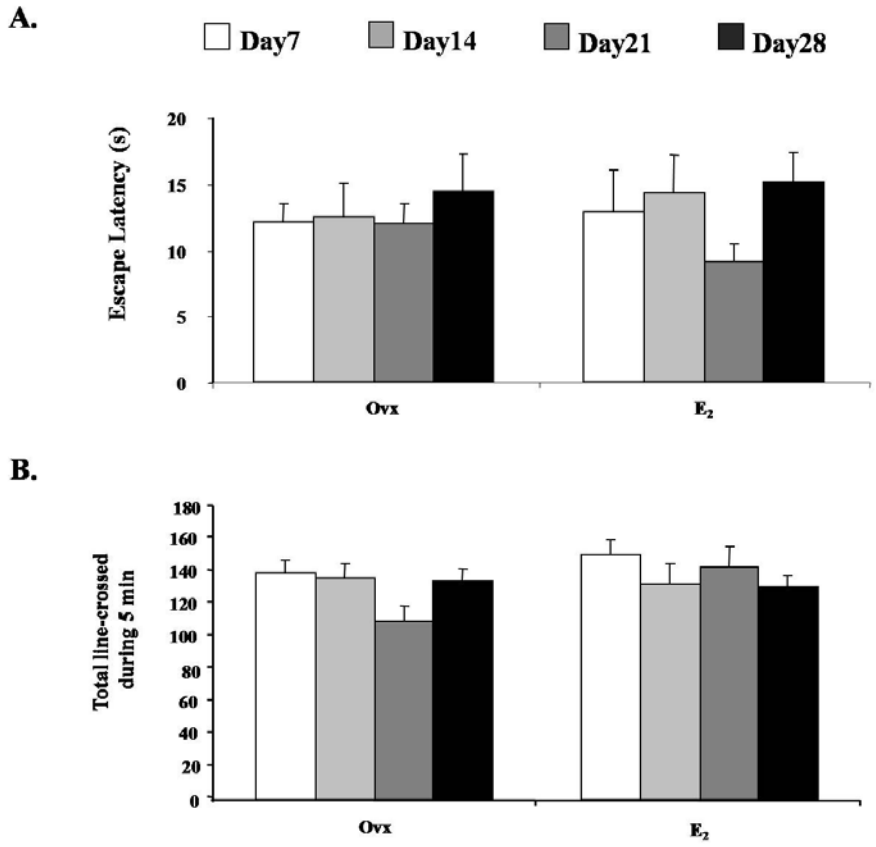

Figure 4-2 The effects of time of estrogen deprivation on (A) escape latency in ETM and (B) locomotor activity in the open field**.** Data present as mean $\pm$ SEM. n = 10-12 for each subgroup.

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.

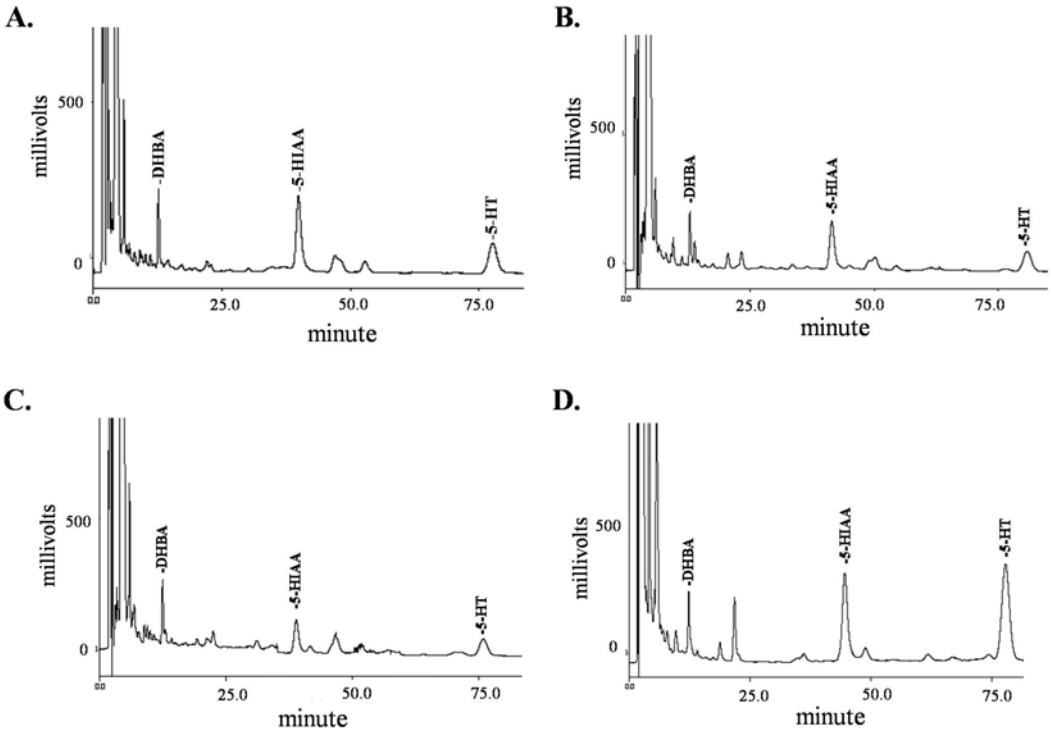

Figure 4-3 The chromatograms represent 5-HT and 5-HIAA levels in midbrain of (A) Ovx7, (B) Ovx14, (C) Ovx21 and (D) Ovx28 groups measured by HPLC-EC. The retention times of 5-HIAA and 5-HT were approximately 37.75 and 75.41, respectively.

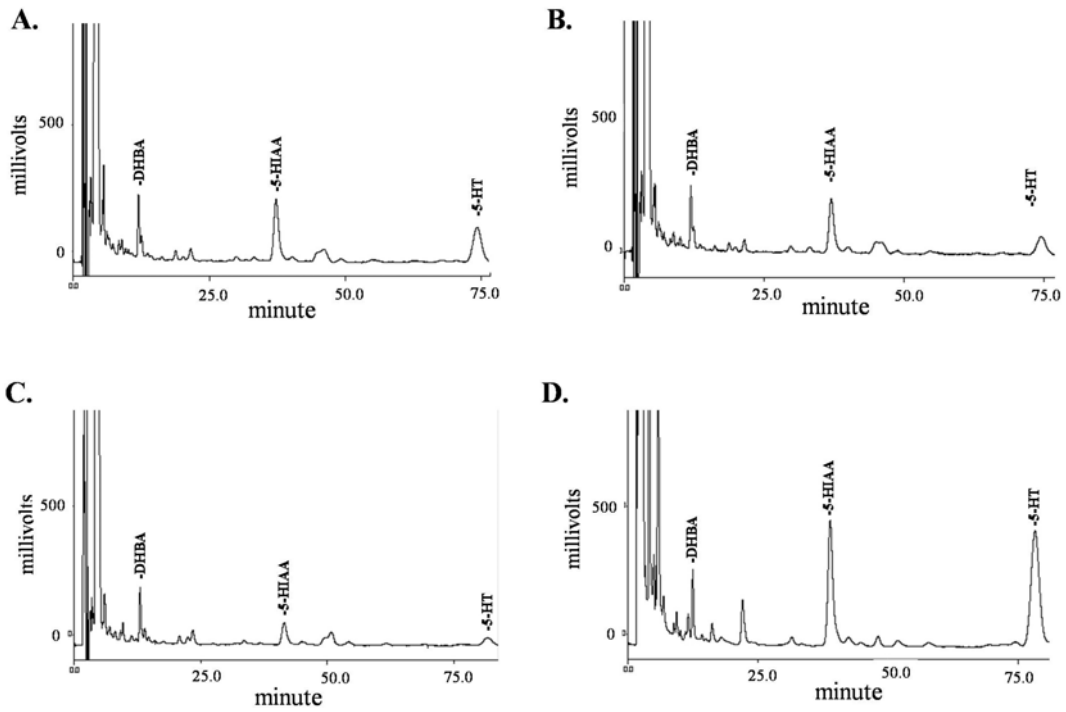

Figure 4-4 The chromatograms represent 5-HT and 5-HIAA levels in midbrain of (A) E7, (B) E14, (C) E21 and (D) E28 groups measured by HPLC-EC. The retention times of 5-HIAA and 5-HT were approximately 37.75 and 75.41, respectively.

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.

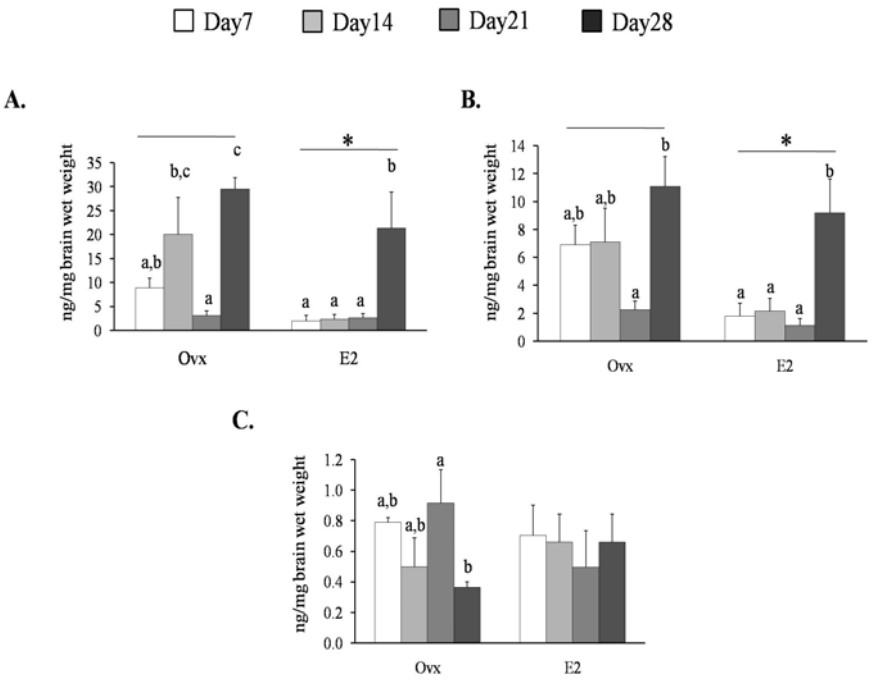

Figure 4-5 The effect of time of estrogen deprivation on (A) 5-HT, (B) 5-HIAA levels and (C) 5-HIAA/5-HT ratios in midbrain. Data presented as mean ± SEM; *P< 0.05 significant difference from Ovx groups, two-way ANOVA. ^{a,b,c} Different letters denoted significant different at P< 0.05, one-way ANOVA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

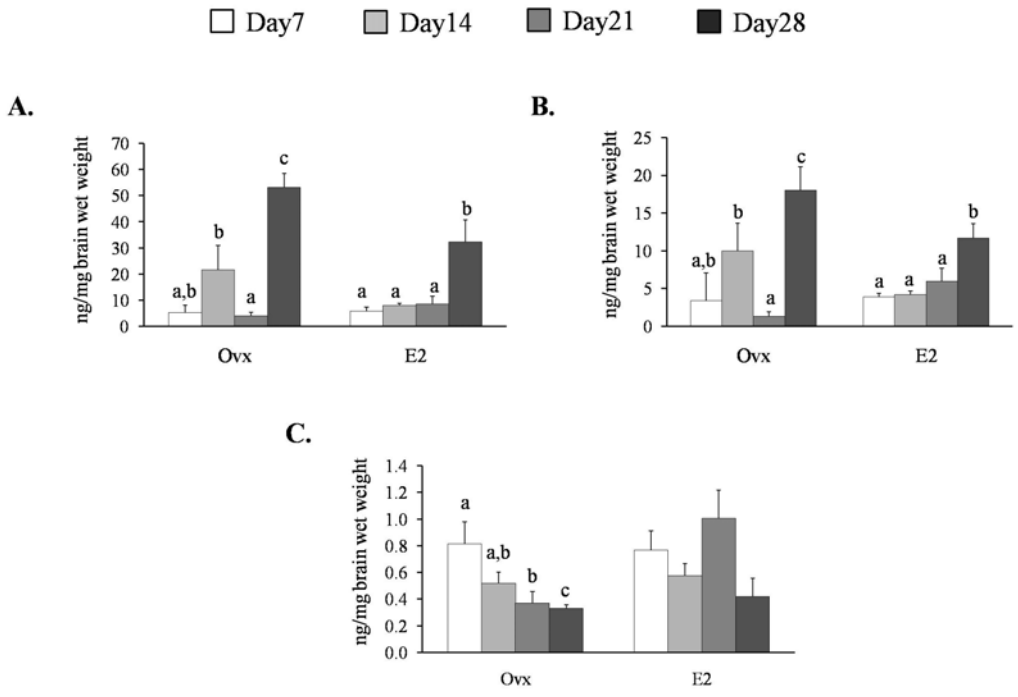

Figure 4-6 The effect of time of estrogen deprivation on (A) 5-HT, (B) 5-HIAA levels and (C) 5-HIAA/5-HT ratios in amygdala. Data presented as mean ± SEM; *P< 0.05 significant difference from Ovx groups, two-way ANOVA. [a,b,c] Different letters denoted significant different at P< 0.05, one-way ANOVA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

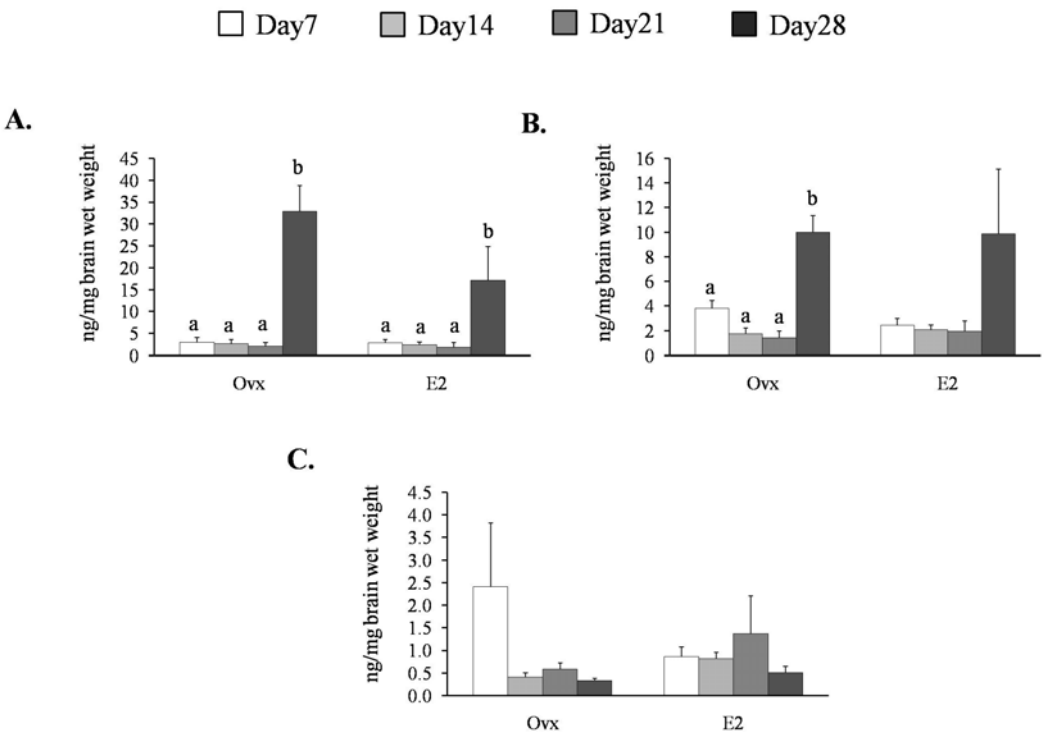

Figure 4-7 The effect of time of estrogen deprivation on (A) 5-HT, (B) 5-HIAA levels and (C) 5-HIAA/5-HT ratios in frontal cortex. Data presented as mean ± SEM; [a,b,c] Different letters denoted significant different at P< 0.05, one-way ANOVA followed by Duncan's multiple comparison test. n = 6 for each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

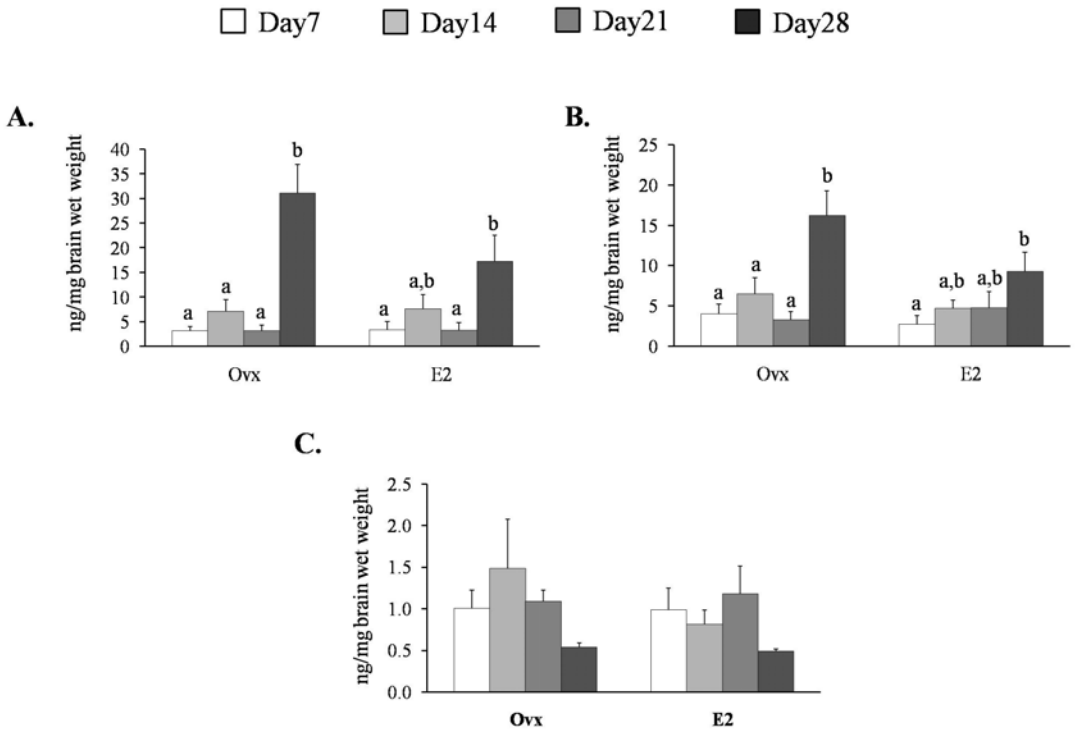

Figure 4-8 The effect of time of estrogen deprivation on (A) 5-HT, (B) 5-HIAA levels and (C) 5-HIAA/5-HT ratios in hippocampus. Data presented as mean ± SEM; ^{a,b,c} Different letters denoted significant different at P< 0.05, one-way ANOVA followed by Duncan's multiple comparison test. n = 6 for each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

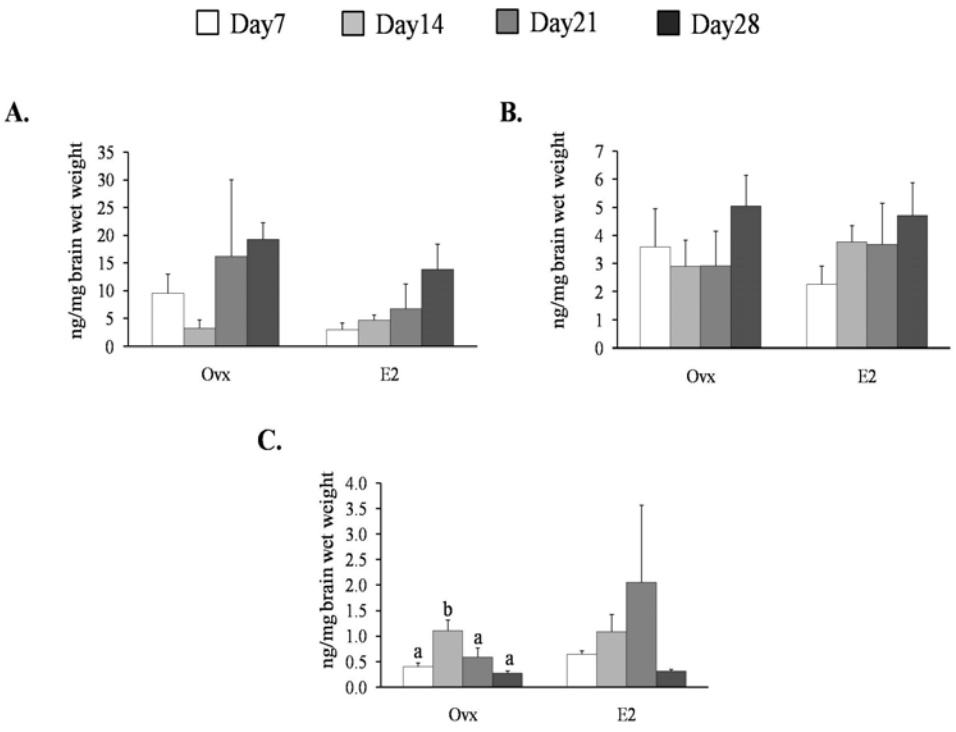

Figure 4-9 The effect of time of estrogen deprivation on (A) 5-HT, (B) 5-HIAA levels and (C) 5-HIAA/5-HT ratios in nucleus accumbens. Data presented as mean $\pm$ SEM; ^{a,b,c} Different letters denoted significant different at $P < 0.05$, ANOVA followed by Duncan's multiple comparison test. n = 6 for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

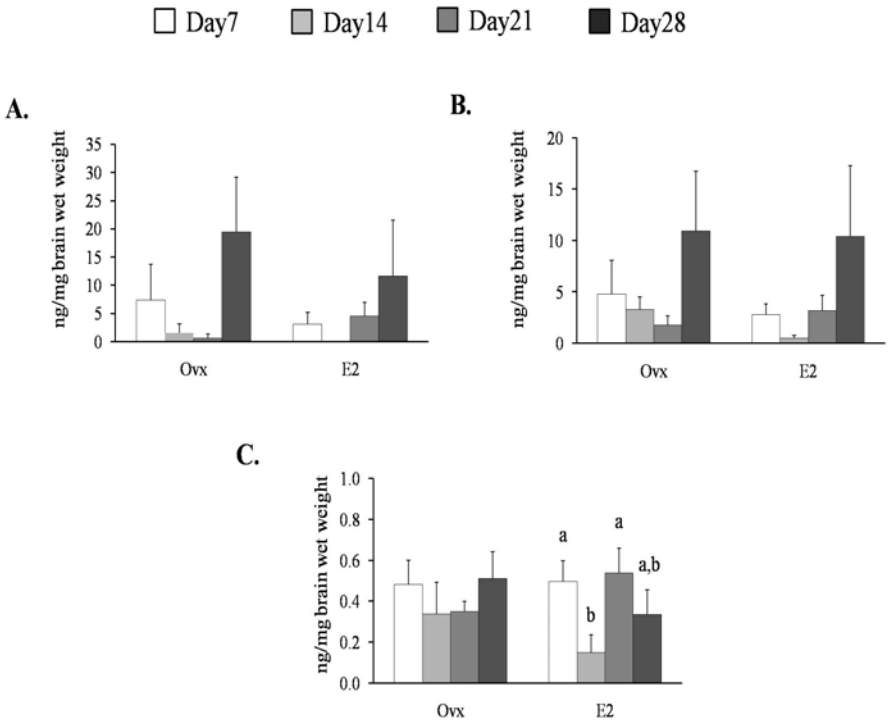

Figure 4-10 The effect of time of estrogen deprivation on (A) 5-HT, (B) 5-HIAA levels and (C) 5-HIAA/5-HT ratios in septum. Data presented as mean $\pm$ SEM; ^{a,b,c} Different letters denoted significant different at $P < 0.05$, ANOVA followed by Duncan's multiple comparison test. n = 6 for each subgroup.

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.

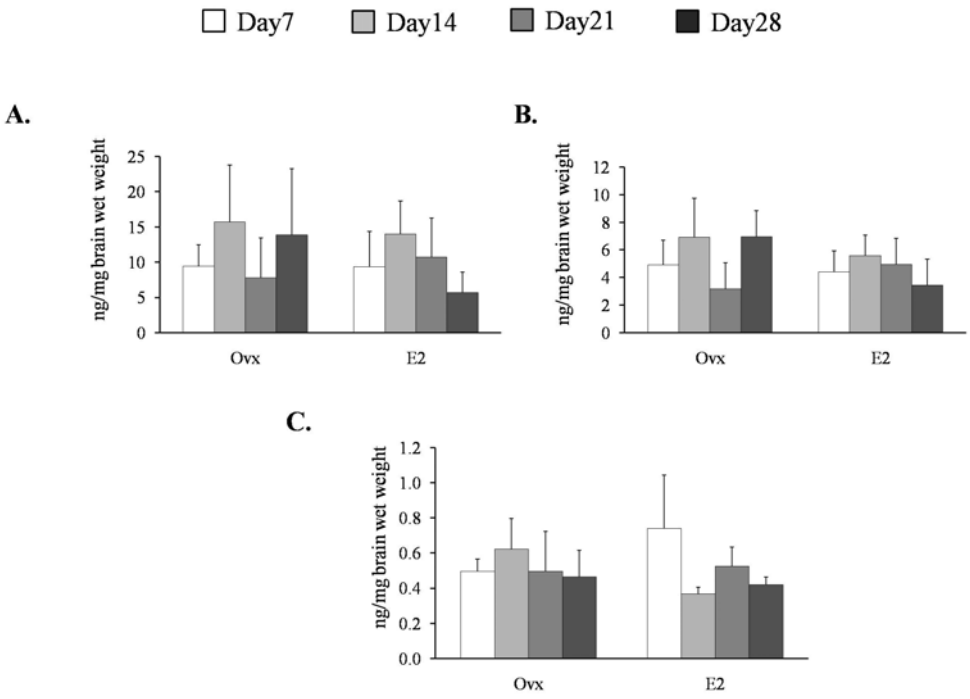

Figure 4-11 The effect of time of estrogen deprivation on (A) 5-HT, (B) 5-HIAA levels and (C) 5-HIAA/5-HT ratios in anterior hypothalamus. Data presented as mean $\pm$ SEM; n = 6 for each subgroup.

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.

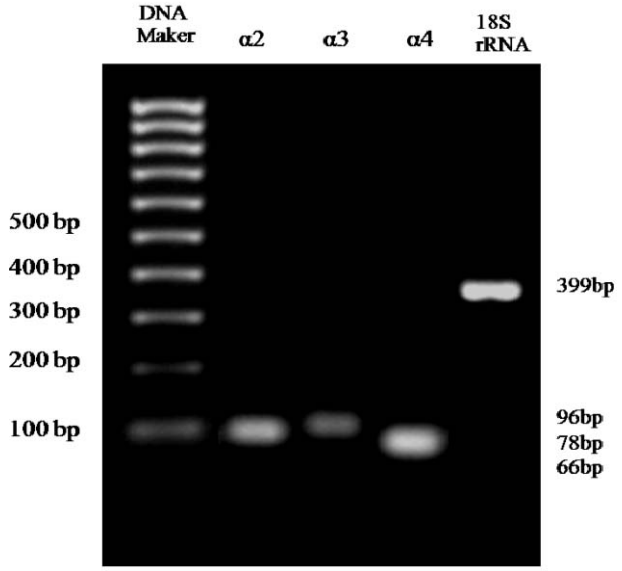

Figure 4-12 Agarose gel electrophoresis of PCR amplification products of $GABA_A$ receptor α2, α3, α4 subunits and 18s rRNA. The numbers on the left indicate the DNA ladder; while, the numbers on the right indicate the size(s) of each PCR product.

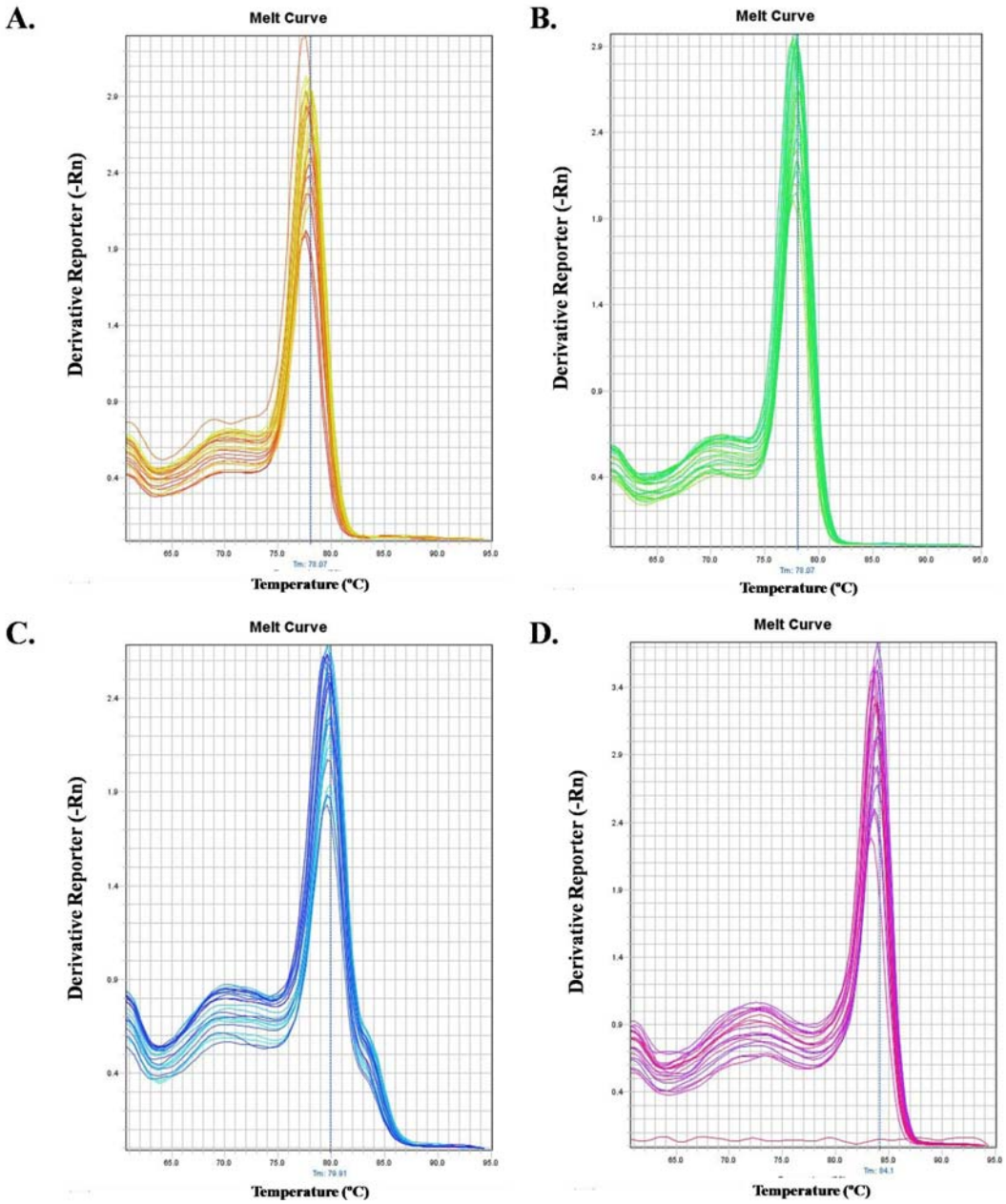

Figure 4-13 Dissociation curves of PCR amplification products of $GABA_A$ receptor (A) α2, (B) α3, (C) α4 subunits and (D) 18s rRNA.

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.

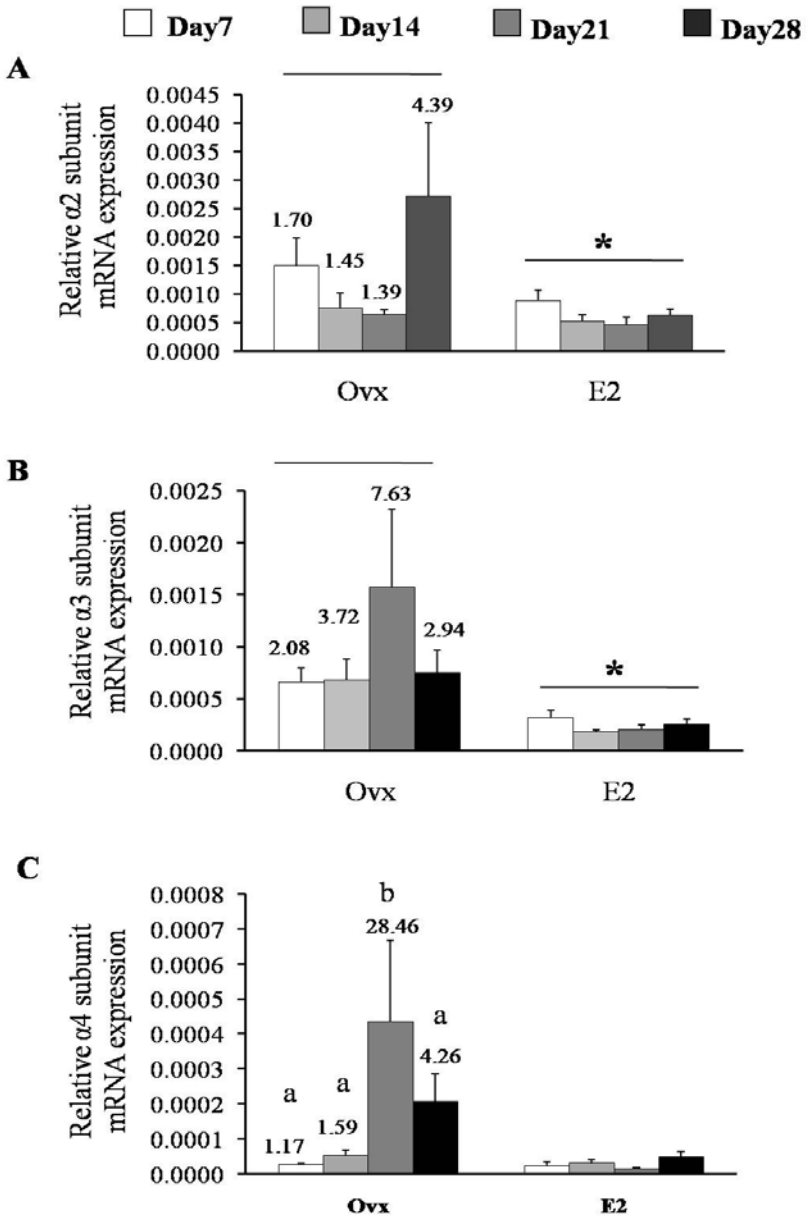

Figure 4-14 The effect of time of estrogen deprivation on $GABA_A$ receptor (A) α2-, (B) α3- and (C) α4- subunit gene expressions in midbrain. Data presented as mean ± SEM; * significant difference ($P < 0.05$) from Ovx groups, two-way ANOVA. [a,b] Different letters denoted significant different within 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.

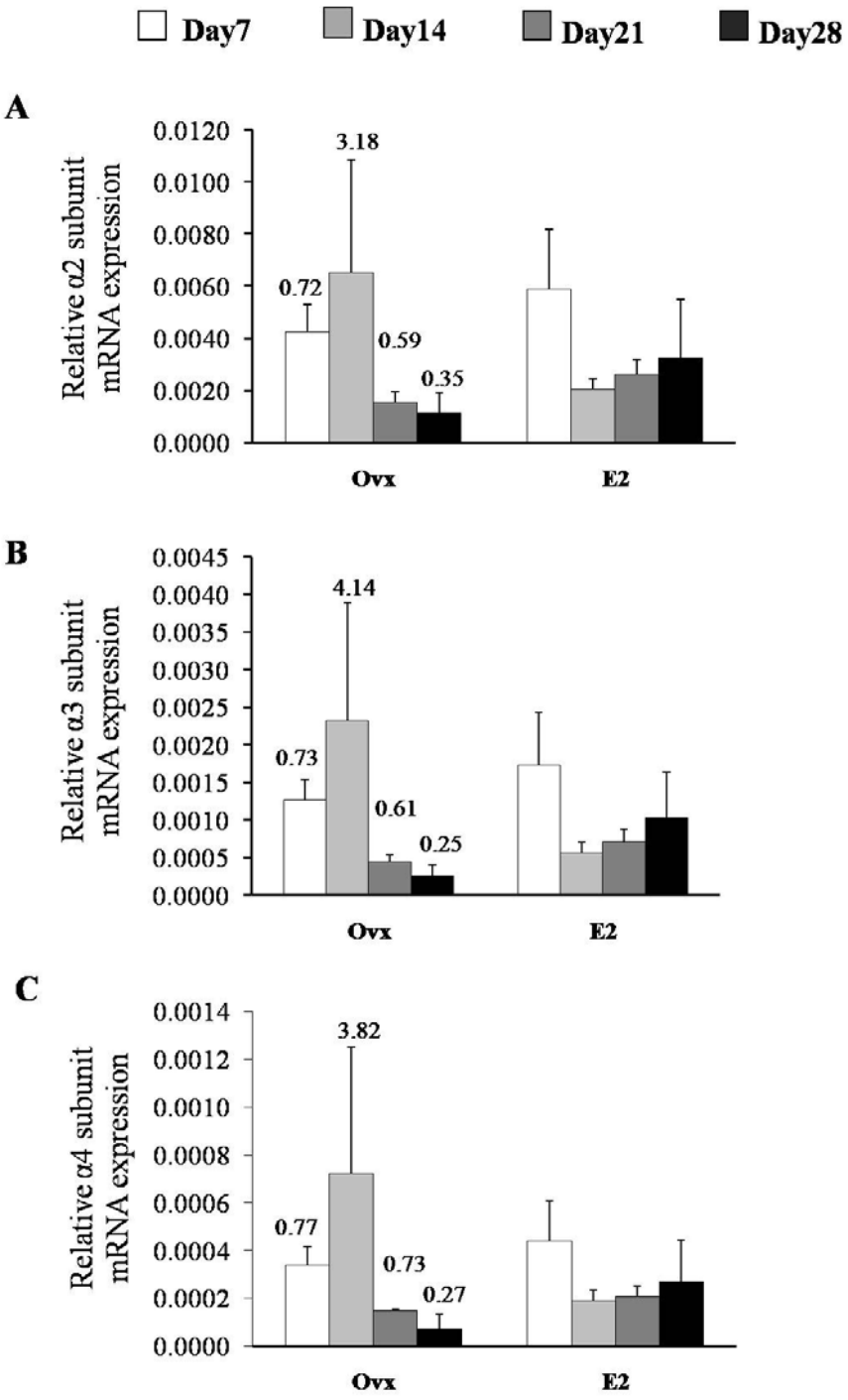

Figure 4-15 The effect of time of estrogen deprivation on $GABA_A$ receptor (A) α2-, (B) α3- and (C) α4- subunit gene expressions in amygdala. Data presented as mean ± SEM; the number above each bar represents fold change from E_2 at the same time point. n = 3 for each subgroup.

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).

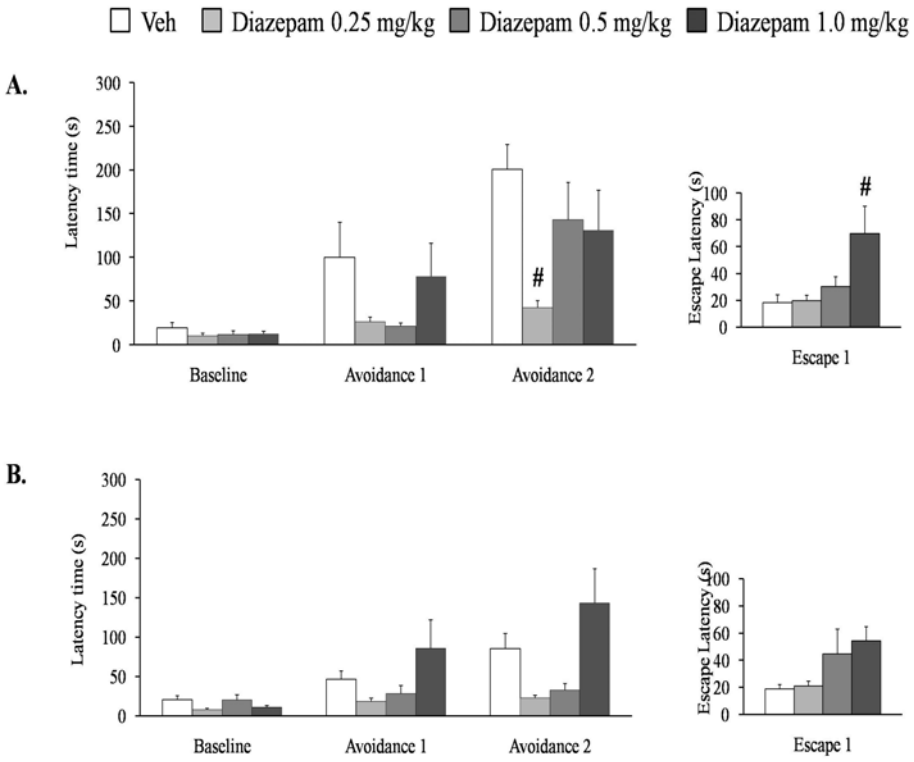

Figure 4-16 The effect of benzodiazepine agonist on anxiety-like behavior in the ETM of the (A) Ovx and (B) E_2 groups. The figures on the left panel are inhibitory avoidance trials and the figures on the right panel are escape trials. Data presented as mean $\pm$ SEM; $^{\#}$ $P<0.05$ compared to vehicle 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.

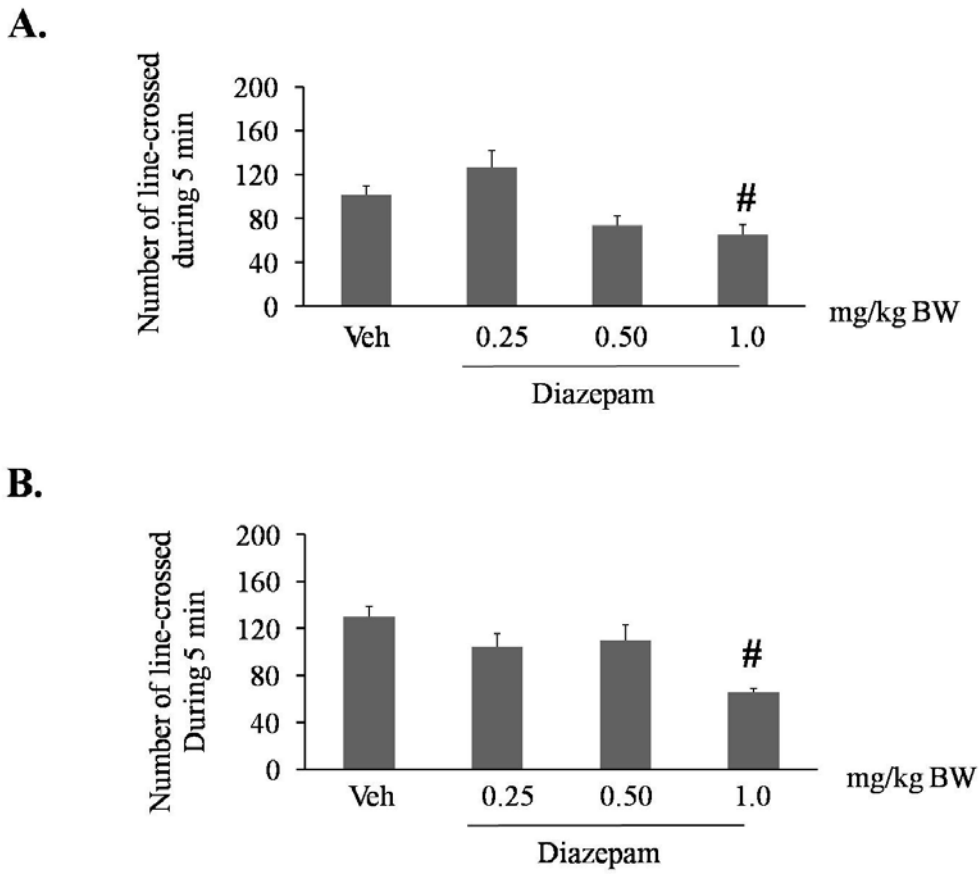

Figure 4-17 The effect of benzodiazepine agonist on locomotor activity in (A) Ovx groups and (B) E_2 groups. Data presented as mean $\pm$ SEM; $^{\#}$ P<0.05 compared to vehicle treated Ovx or E_2 rats, ANOVA followed by Dunnett's test, n= 9-10 for each subgroup.

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).

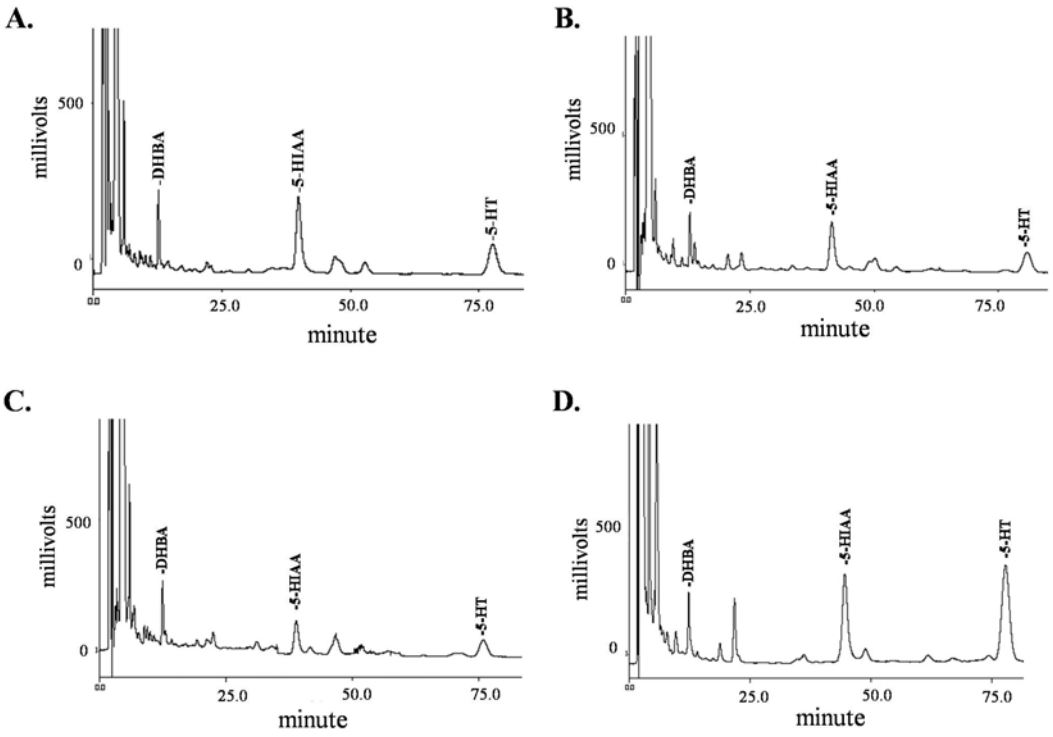

Figure 4-18 The chromatograms represent 5-HT and 5-HIAA levels in midbrain of (A) vehicle-, (B) 0.25 mg/kg diazepam-, (C) 0.5 mg/kg diazepam- and (D) 1.0 mg/kg diazepam- treated Ovx rats as measured by HPLC-EC. The retention times of 5-HIAA and 5-HT were approximately 37.75 and 75.41, respectively.

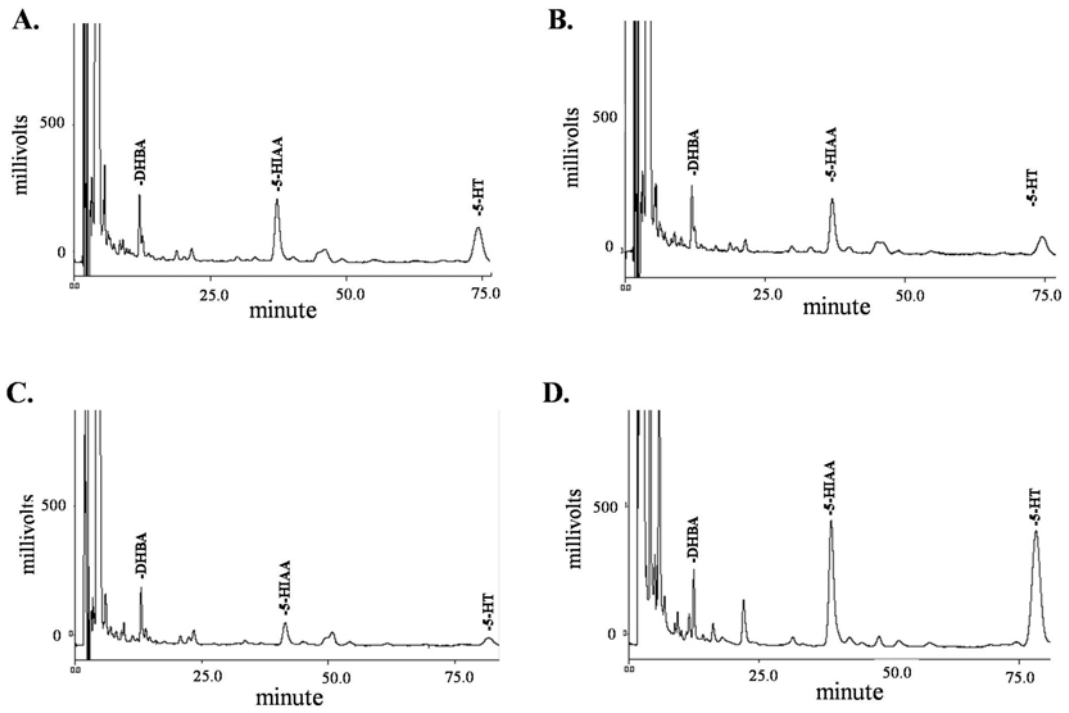

Figure 4-19 The chromatograms represent 5-HT and 5-HIAA levels in midbrain of (A) vehicle-, (B) 0.25 mg/kg diazepam-, (C) 0.5 mg/kg diazepam- and (D) 1.0 mg/kg diazepam- treated E_2 rats as measured by HPLC-EC. The retention times of 5-HIAA and 5-HT were approximately 37.75 and 75.41, respectively.

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.

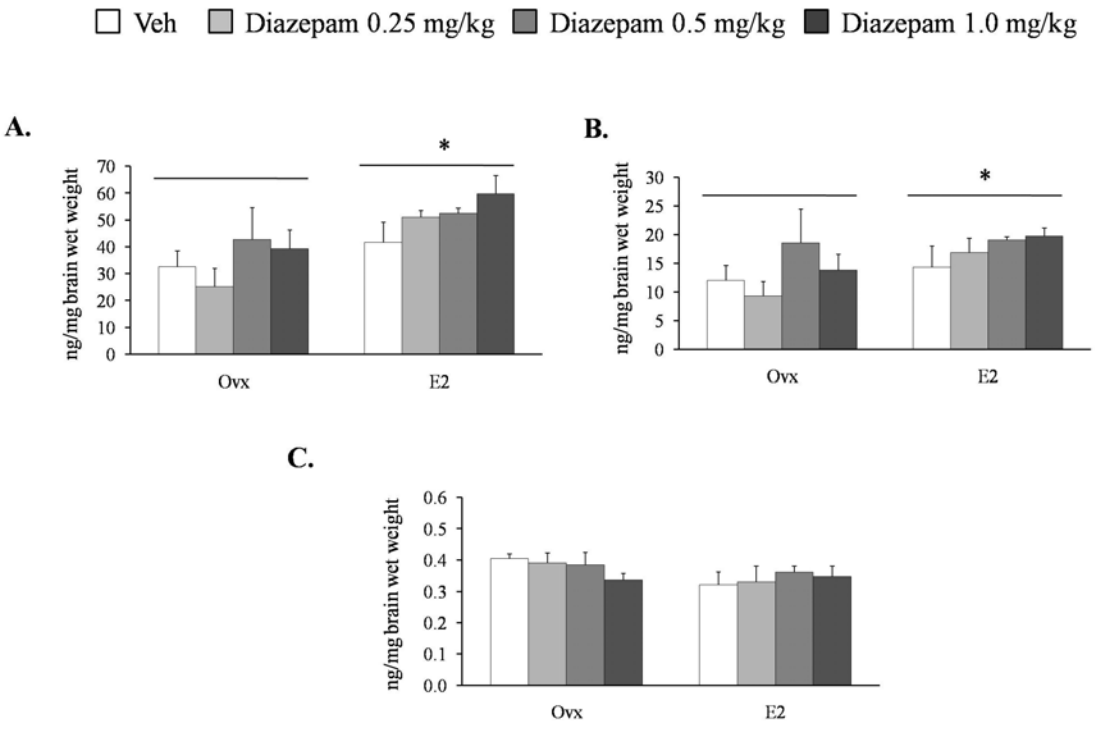

Figure 4-20 The effects of benzodiazepine agonist on (A) 5-HT, (B) 5-HIAA levels and (C) 5-HIAA/5-HT ratios in the midbrain. Data presented as mean ± SEM; * P < 0.05, significant difference from Ovx groups, two-way ANOVA. n = 6-7 for each subgroup.

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.

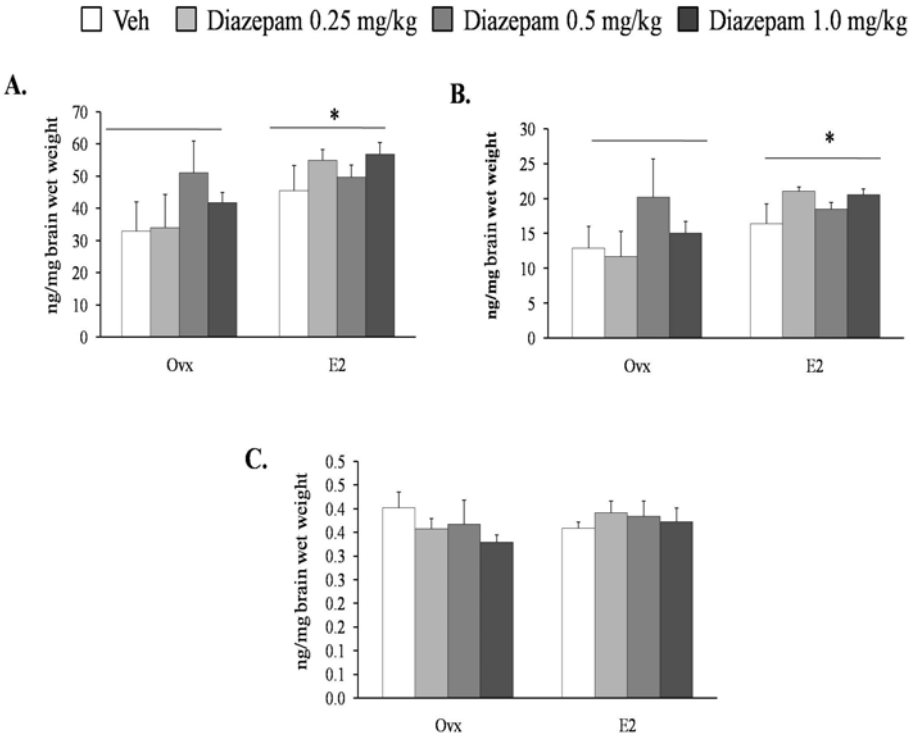

Figure 4-21 The effects of benzodiazepine agonist on (A) 5-HT, (B) 5-HIAA levels and (C) 5-HIAA/5-HT ratios in the amygdala. Data presented as mean ± SEM; * P < 0.05, significant difference from Ovx groups, two-way ANOVA. n = 6-7 for each subgroup.

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).

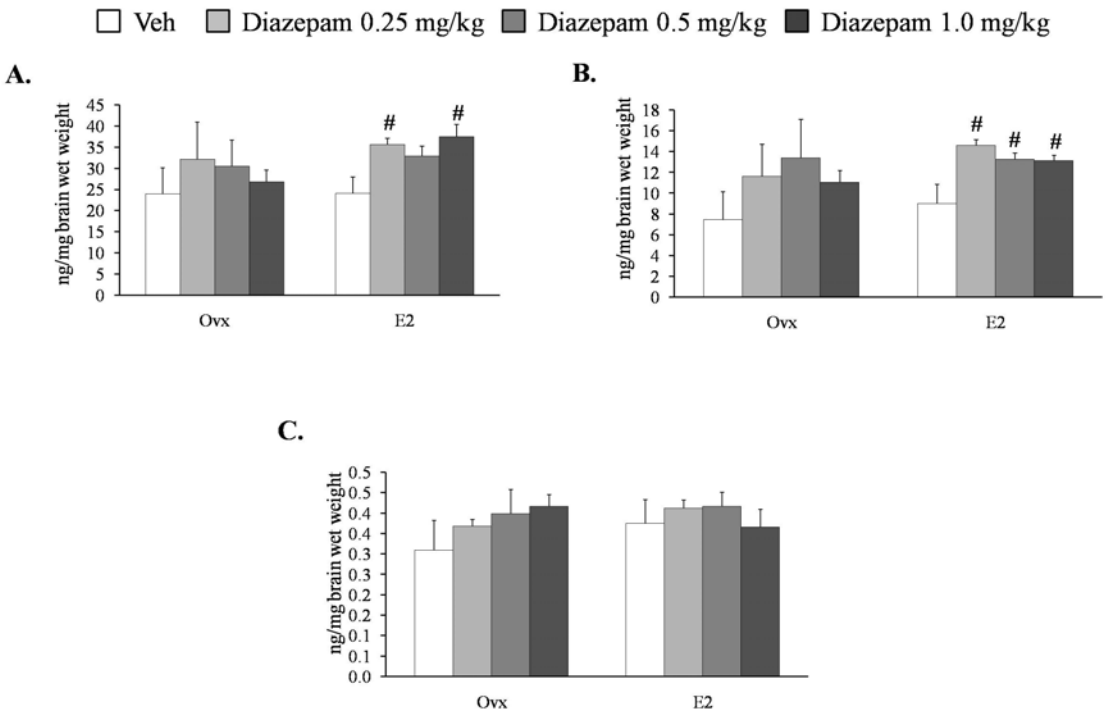

Figure 4-22 The effects of benzodiazepine agonist on (A) 5-HT, (B) 5-HIAA levels and (C) 5-HIAA/5-HT ratios in the frontal cortex. Data presented as mean ± SEM; # P < 0.05, significant difference from vehicle treated rats within the same group, ANOVA followed by Dunnett's test, n = 6-7 for each subgroup.

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.

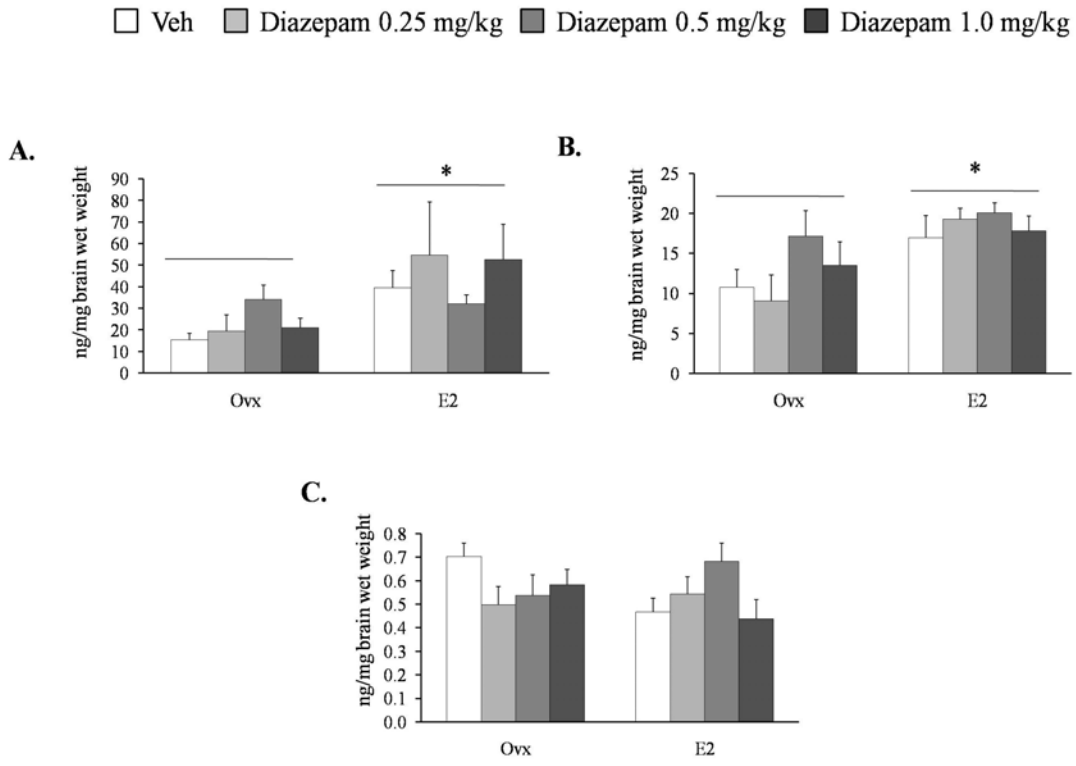

Figure 4-23 The effects of benzodiazepine agonist on (A) 5-HT, (B) 5-HIAA levels and (C) 5-HIAA/5-HT ratios in the hippocampus. Data presented as mean ± SEM; * P < 0.05, significant difference from Ovx groups, two-way ANOVA. n = 6-7 for each subgroup.

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).

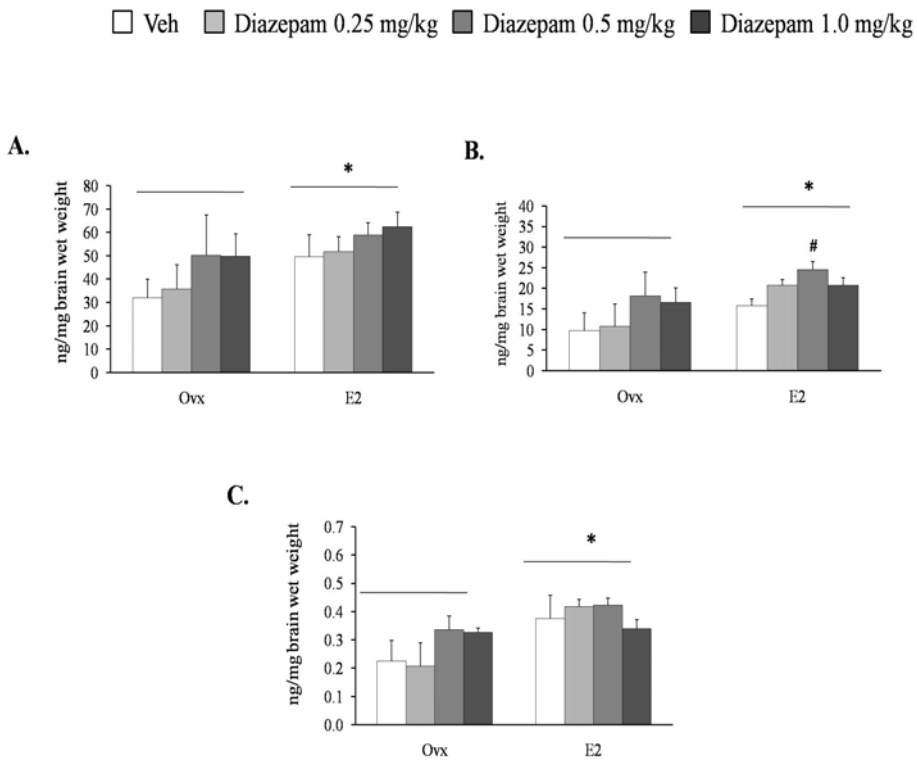

Figure 4-24 The effects of benzodiazepine agonist on (A) 5-HT, (B) 5-HIAA levels and (C) 5-HIAA/5-HT ratios in the nucleus accumbens. Data presented as mean ± SEM; * $P < 0.05$, significant difference from Ovx groups, two-way ANOVA. n = 6-7 for each group. $^{\#}P < 0.05$, significant difference from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

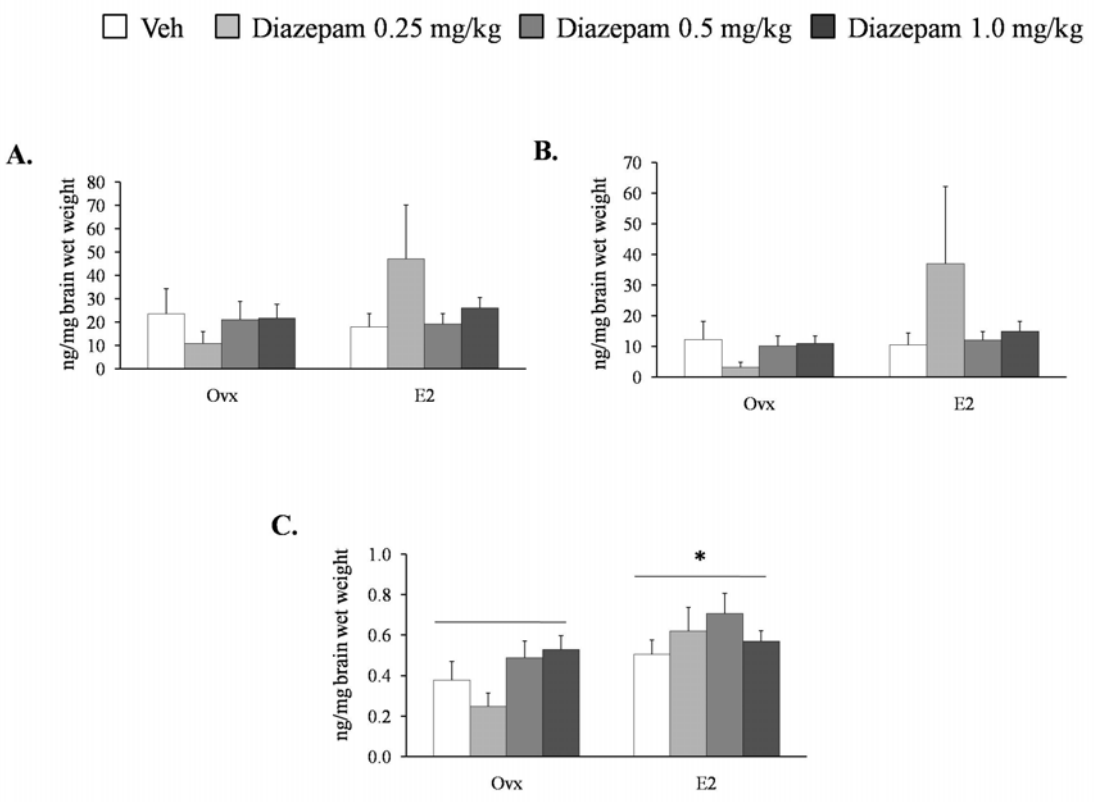

Figure 4-25 The effects of benzodiazepine agonist on (A) 5-HT, (B) 5-HIAA levels and (C) 5-HIAA/5-HT ratios in the septum. Data presented as mean ± SEM; * P < 0.05, significant difference from Ovx groups, two-way ANOVA. n = 6-7 for each group.

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.

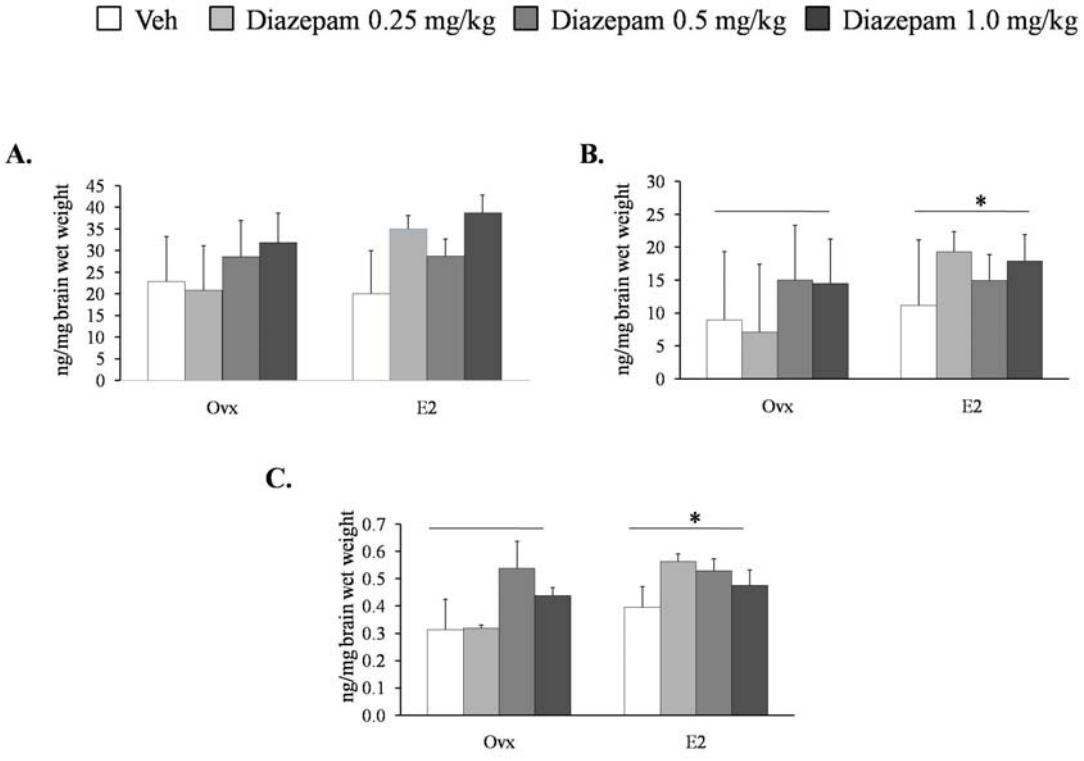

Figure 4-26 The effects of benzodiazepine agonist on (A) 5-HT, (B) 5-HIAA levels and (C) 5-HIAA/5-HT ratios in the anterior hypothalamus. Data presented as mean $\pm$ SEM; * $P < 0.05$, significant difference from Ovx groups, two-way ANOVA. n = 6-7 for each group.

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 ($P > 0.05$).

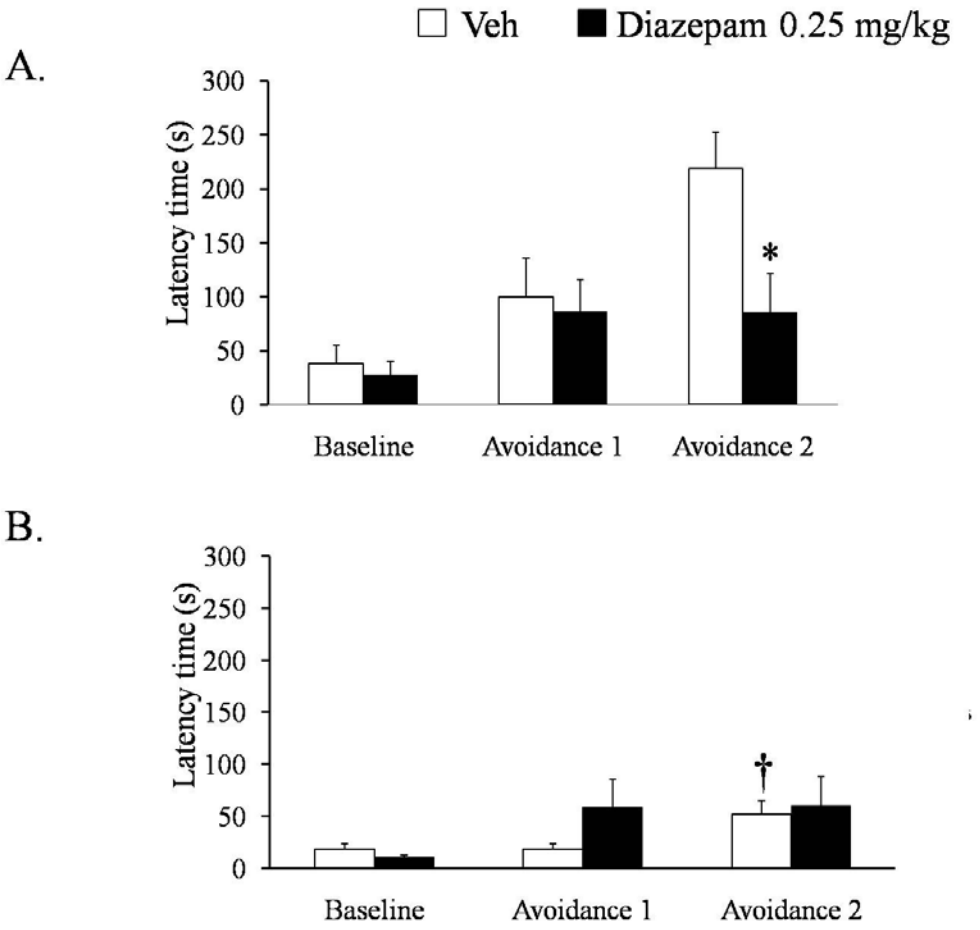

Figure 4-27 The effects of benzodiazepine agonist on anxiety-like behavior after long-term ovariectomy in (A) Ovx and (B) E_2 rats. Data presented as mean ± S.E.M., * $P < 0.05$, compared with vehicle treated rats within the same group; † $P < 0.05$ compared between Ovx and E_2 group with the same treatment 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.

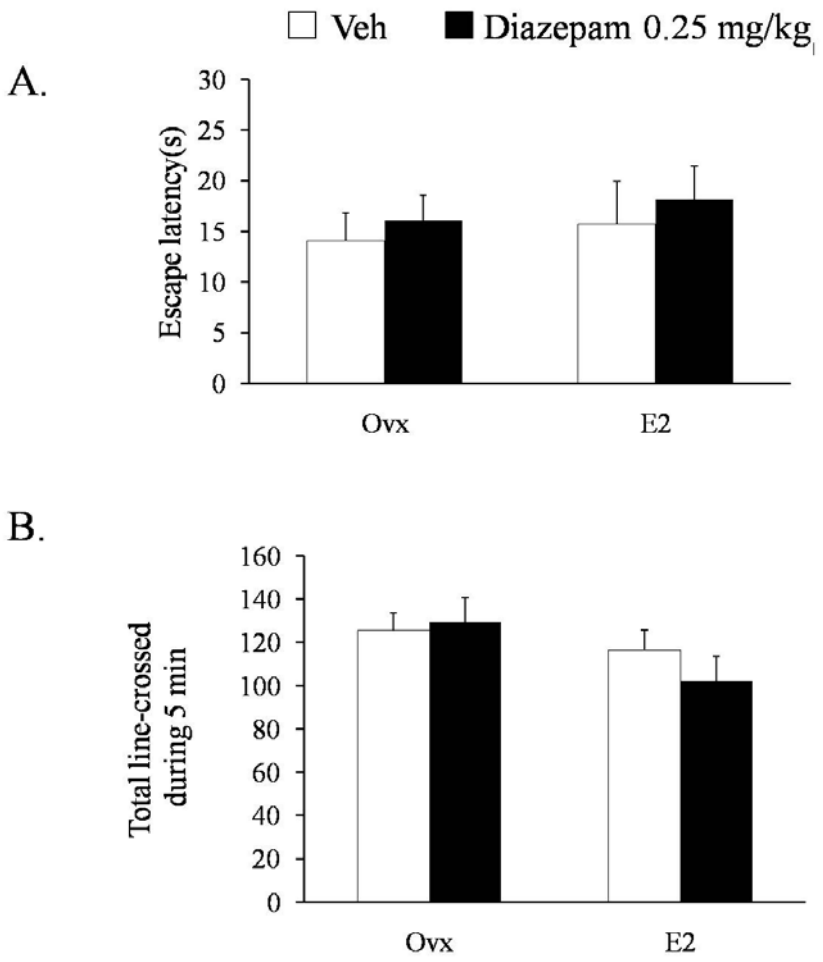

Figure 4-28 The effects of benzodiazepine agonist on (A) escape behavior and (B) locomotor activity in the Ovx and E_2 rats after long-term ovariectomy. Data presented as mean $\pm$ S.E.M. n = 9-10 to each subgroup.

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.

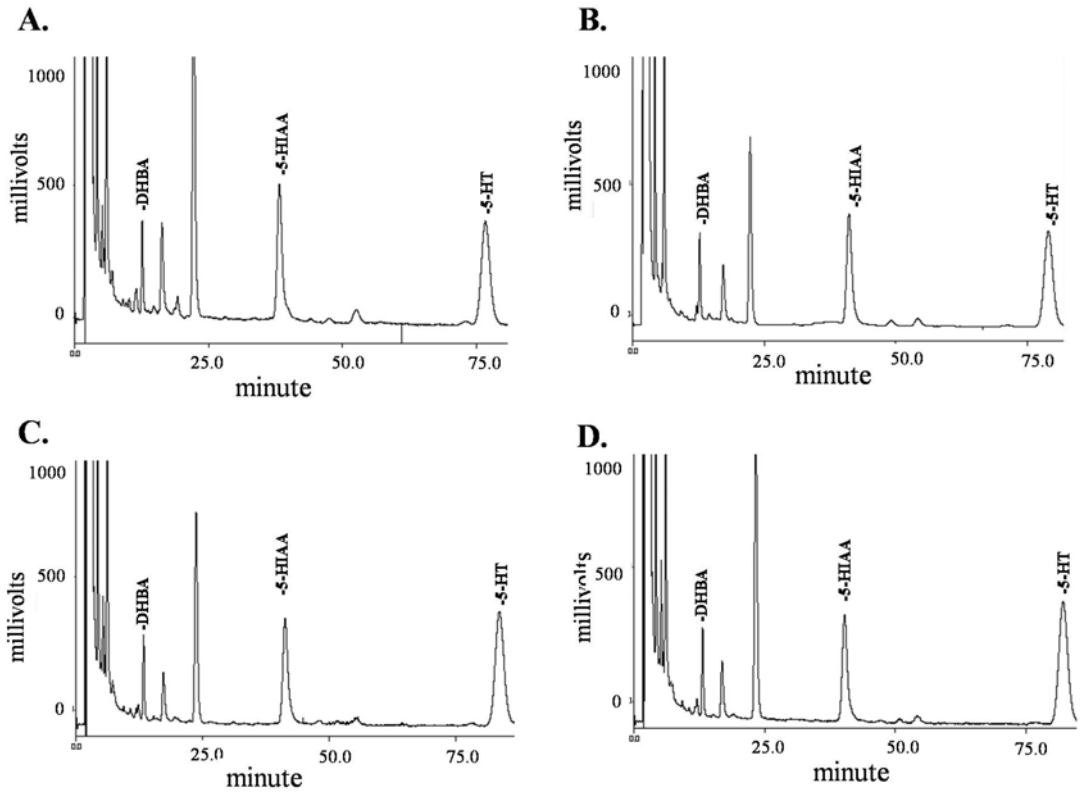

Figure 4-29 The chromatograms represent 5-HT and 5-HIAA levels in midbrain of (A) Ovx rats treated with vehicle, (B) Ovx rats treated with diazepam, (C) E_2 rats treated with vehicle and (D) E_2 rats treated with diazepam as measured by HPLC-EC. The retention times of 5-HIAA and 5-HT levels were approximately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

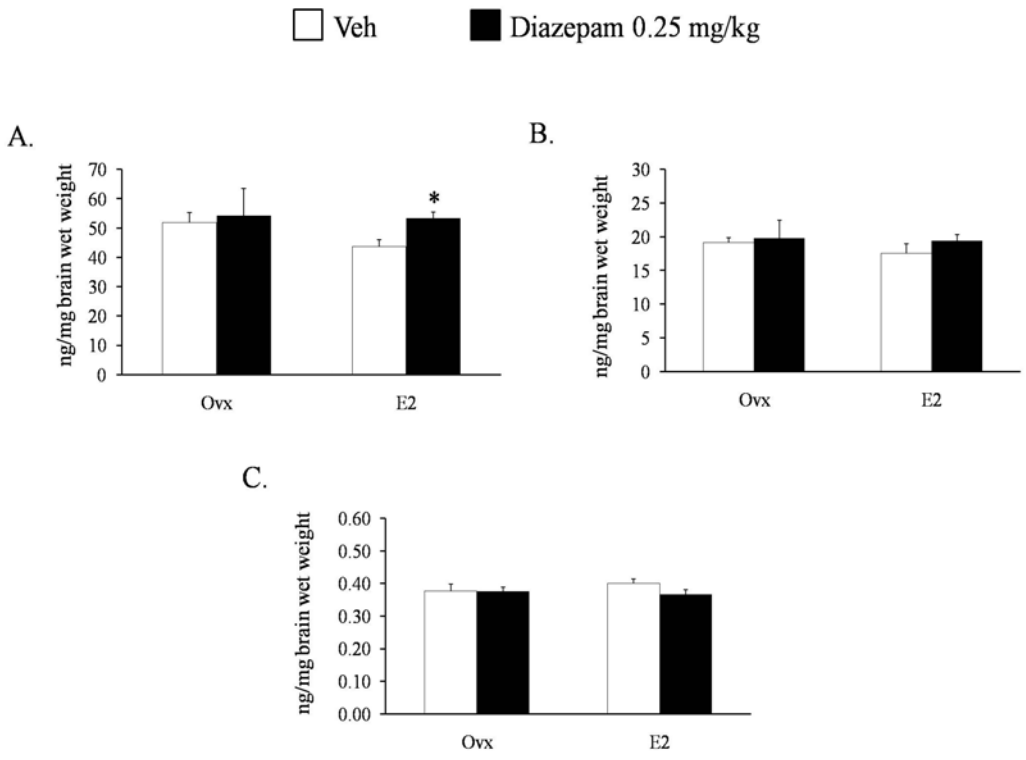

Figure 4-30 The effects of benzodiazepine agonist on the levels of (A) 5-HT, (B) HIAA and 5-HIAA/5-HT ratio in midbrain. * P<0.05, significantly different from vehicle treated rats within the same group using Student's unpaired *t*-test. n = 8 to each subgroup.

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.

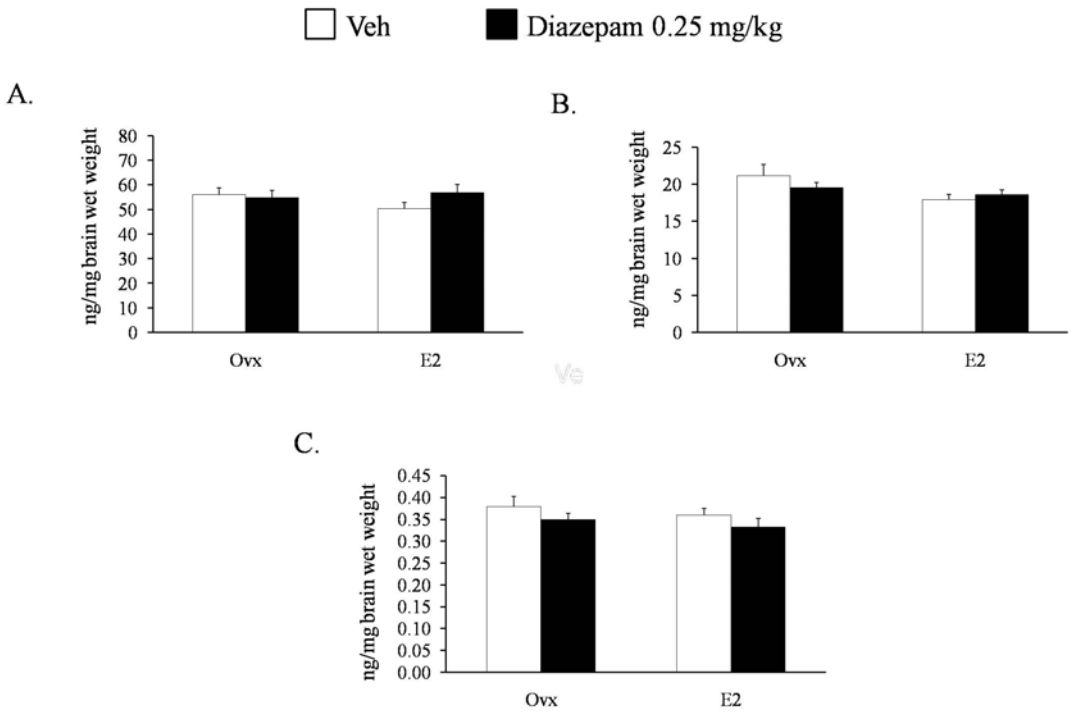

Figure 4-31 The effects of benzodiazepine agonist on the levels of (A) 5-HT, (B) HIAA and 5-HIAA/5-HT ratio in amygdala. n = 8 to each group.

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.

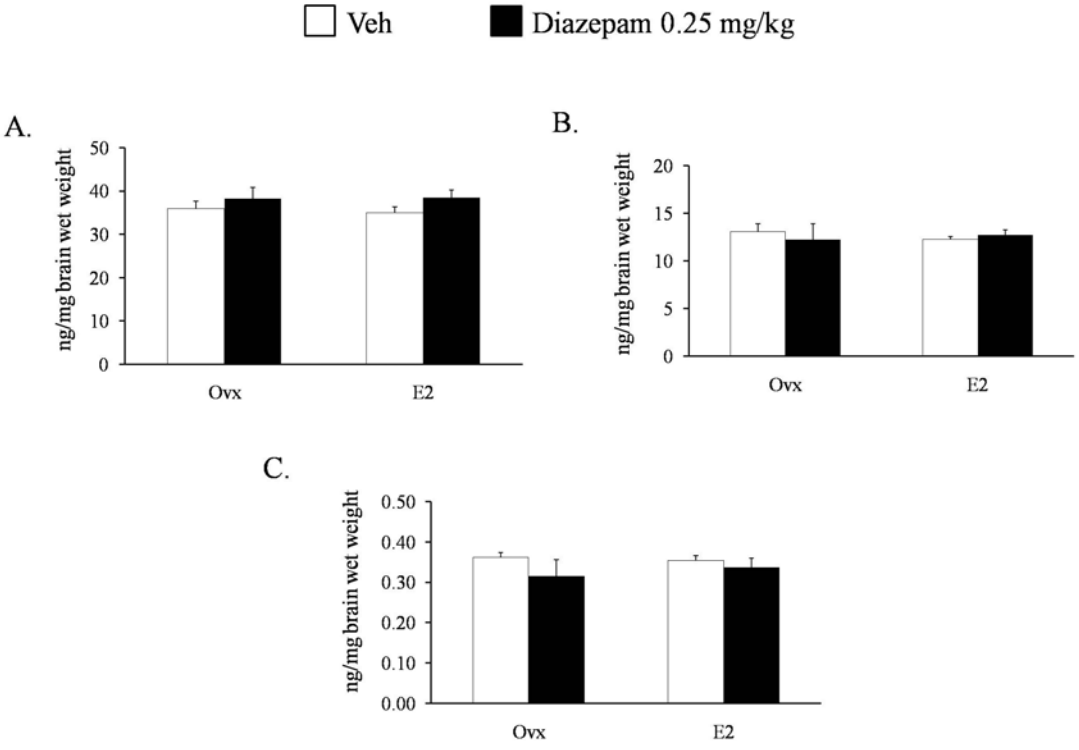

Figure 4-32 The effects of benzodiazepine agonist on the levels of (A) 5-HT, (B) HIAA and 5-HIAA/5-HT ratio in frontal cortex. n = 8 to each subgroup.

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).

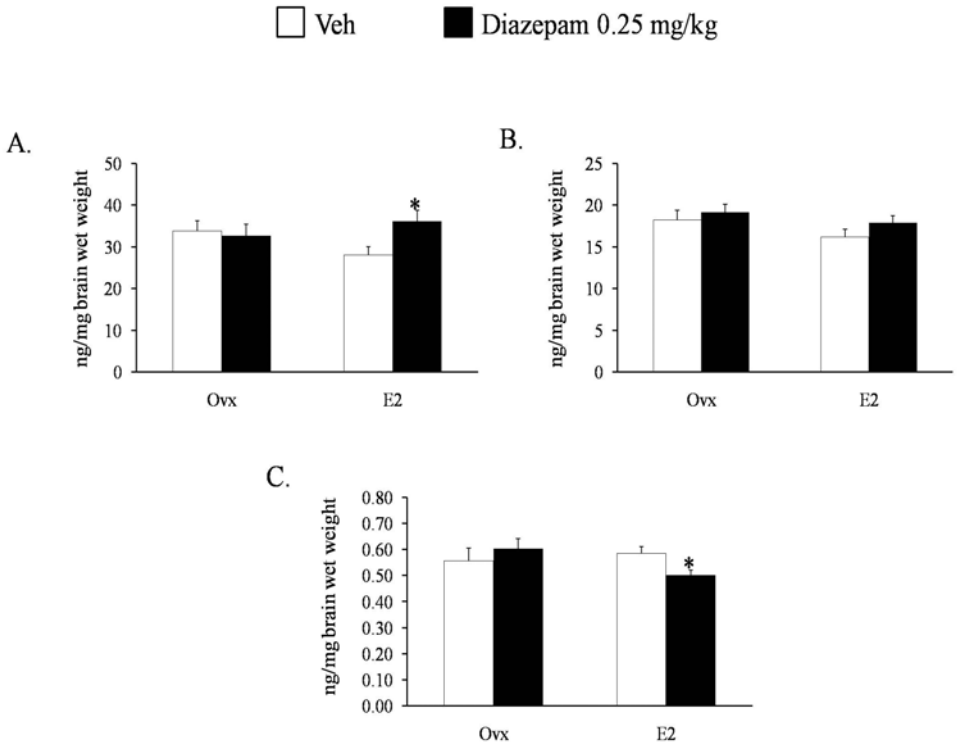

Figure 4-33 The effects of benzodiazepine agonist on the levels of (A) 5-HT, (B) HIAA and 5-HIAA/5-HT ratio in hippocampus. * P<0.05, significantly different from vehicle treated rats within the same group using Student's unpaired *t*-test. n = 8 to each subgroup.

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.

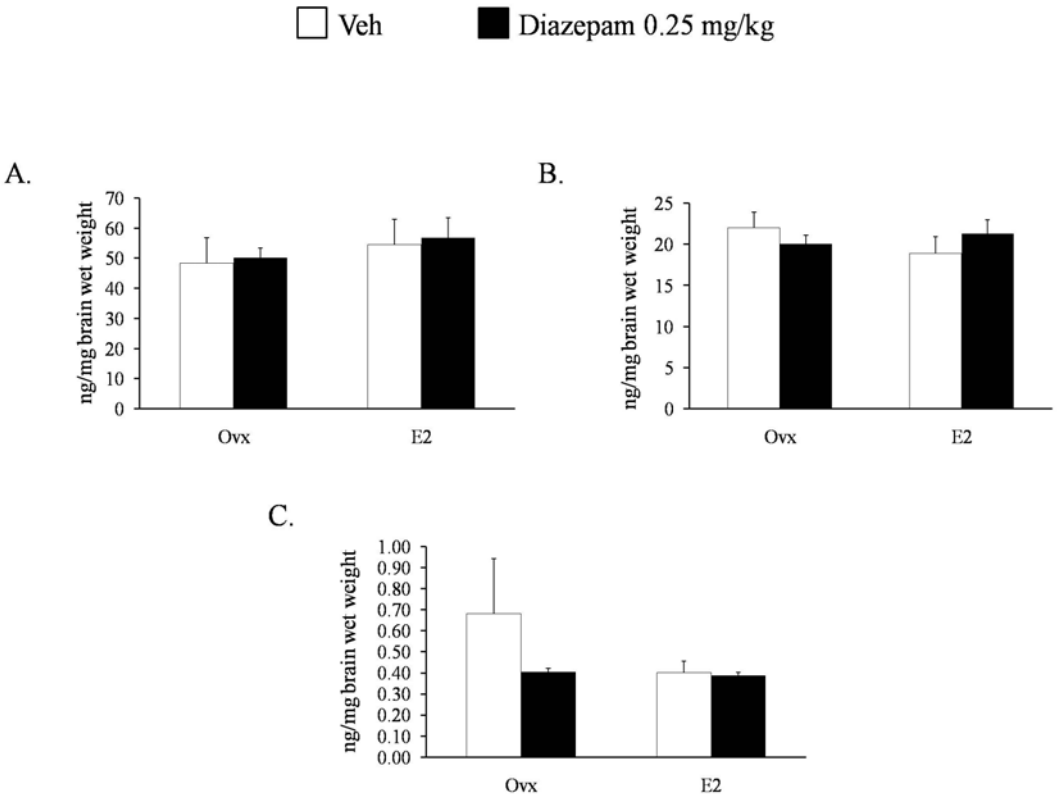

Figure 4-34 The effects of benzodiazepine agonist on the levels of (A) 5-HT, (B) HIAA and 5-HIAA/5-HT ratio in nucleus accumbens. n = 8 to each subgroup.

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).

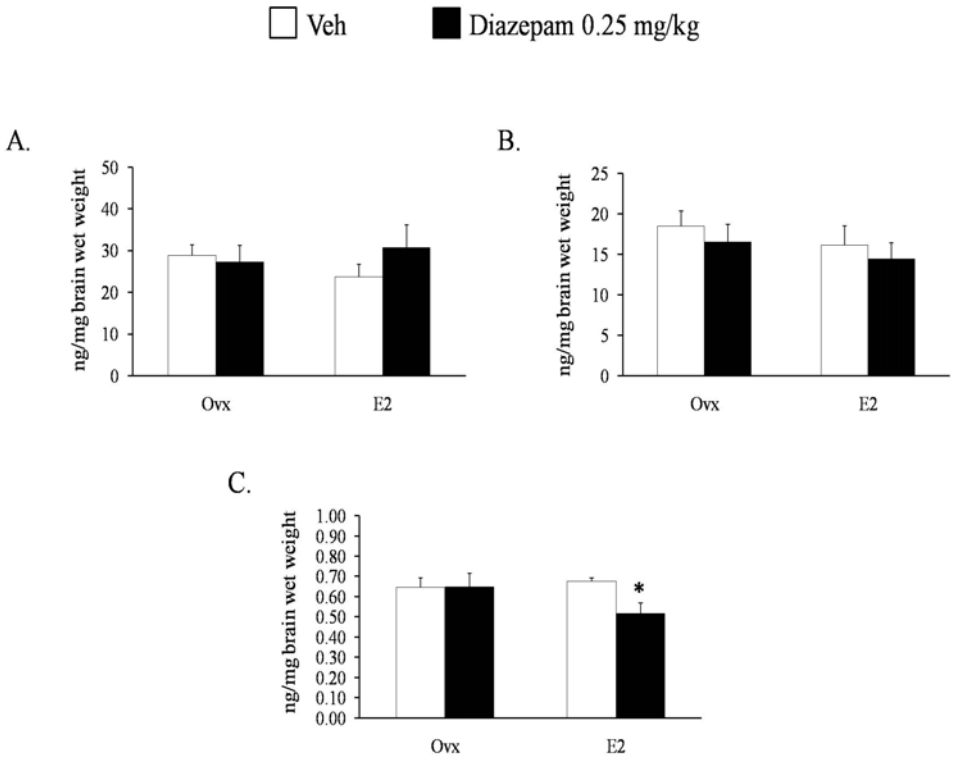

Figure 4-35 The effects of benzodiazepine agonist on the levels of (A) 5-HT, (B) HIAA and 5-HIAA/5-HT ratio in septum. * P<0.05, significantly different from vehicle treated rats within the same group using Student's unpaired *t*-test. n = 8 to each subgroup.

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).

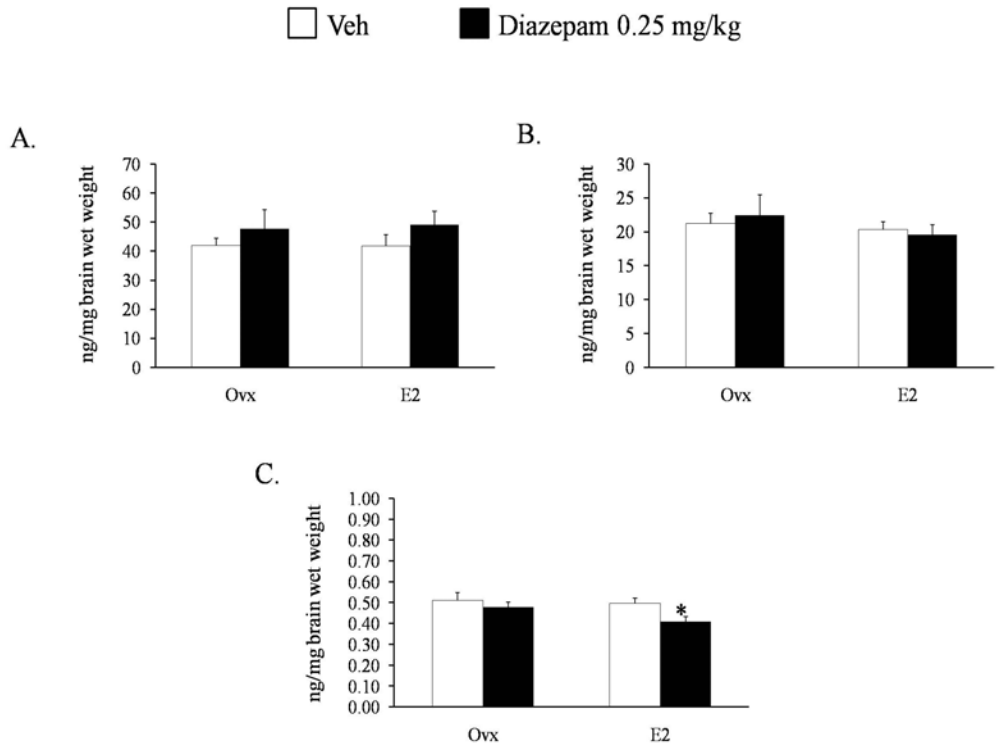

Figure 4-36 The effects of benzodiazepine agonist on the levels of (A) 5-HT, (B) HIAA and 5-HIAA/5-HT ratio in anterior hypothalamus. * P<0.05, significantly different from vehicle treated rats within the same group using Student's unpaired *t*-test. n = 8 to each subgroup.

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.