CHAPTER IV
RESULTS

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

Part 1: The effects of estrogen on the anxiety-like behaviors of the ovariectomized rats in the ETM

Part 2: The effects of estrogen on the serotonergic neurotransmission after the ETM test

Part 3: The effects of estrogen on the postsynaptic serotonin receptor function on the anxiety-like behaviors tested with the ETM

1. The effects of estrogen on the anxiety-like behaviors of the ovariectomized rats in the elevated T-maze

In this experiment, rats were divided into three groups including Ovx, Ovx+E_2, and Pro rats. Four weeks after operations, the rats were tested with ETM. Body weight and food intake were determined daily. Uterine weight in all groups of animals was determined immediately after sacrifice.

1.1. Body weight, food intake, and uterine weight

Table 4 summarizes the mean values ± S.E.M. of initial and final BW, DWG, DFI, UW, and % UW/BW from all groups of experimental animals. Body weights of the animals at the beginning of experiments were not significantly different.

The Ovx rats demonstrated a significantly higher in body weight [$F(2, 107) = 95.04$; $P < 0.001$] and daily weight gain [$F(2, 107) = 156.50$; $P < 0.001$] than those Ovx+E_2 and Pro rats. Moreover, DFI in these Ovx rats was more [$F(2, 107) = 91.64$; $P < 0.001$] than both Ovx+E_2 and Pro rats, significantly.

A significant reduction in UW [$F(2, 107) = 221.74$; $P < 0.001$] and % UW/BW [$F(2, 107) = 209.60$; $P < 0.001$] were clearly demonstrated in Ovx rats, which verified the deprivation of ovarian sex hormones in these animals. Supplementation of Ovx rats with estrogen (10 μg/kg BW, daily) could reverse the suppression of UW in these animals. In addition, Pro rats showed more UW and % UW/BW than Ovx+E_2 rats, significantly.

Table 4 Body weight (BW), daily weight gain (DWG), daily food intake (DFI), uterine weight (UW), and percentage of uterine-to-body weight ratio (% UW/BW) of ovariectomized (Ovx), estrogen treated ovariectomized (Ovx+E_2), and proestrous (Pro) rats.

Parameters	Ovx	Ovx+E_2	Pro
Initial BW (g)	201.12 ± 1.49^b	201.88 ± 1.12^a	200.63 ± 1.59^a
Final BW (g)	284.87 ± 2.44^b	238.61 ± 2.27^a	243.61 ± 3.11^a
DWG (g/day)	2.78 ± 0.08^b	1.19 ± 0.06^a	1.39 ± 0.06^c
DFI (g/day)	15.33 ± 0.16^b	12.95 ± 0.10^a	13.06 ± 0.16^a
UW (g)	0.118 ± 0.002^b	0.363 ± 0.007^a	0.633 ± 0.030^c
UW/BW (%)	0.041 ± 0.001^b	0.152 ± 0.003^a	0.261 ± 0.013^c
Number of rats	36	38	36

Values are mean ± S.E.M. Different letters are significantly different from one other in the same parameters ($P < 0.001$).

1.2. The anxiety-like behaviors in the elevated T-maze

The ETM session consisted of three inhibitory avoidance trials and one-way escape trial held at 30-s intervals. As illustrated in Table 5 and Figure 13A, the inhibitory avoidance in the ETM was impaired in Ovx+E_2 rats. Two way ANOVA showed a significant effect of treatment [$F(2, 30) = 5.19$, $P < 0.05$] and trials [$F(2, 60) = 18.67$, $P < 0.001$]. The Duncan post hoc test showed that in the avoidance 2, the Ovx+E_2 group decreased the latency to leave the enclosed arm, when compared to the Ovx and the Pro rats ($P < 0.05$). Moreover, escape latency from the open arm was significantly increased in the Pro rats [$F(2, 30) = 6.79$, $P < 0.01$] as compared with other groups (Table 5 and Figure 13B).

1.3. The locomotor activity and anxiety-like behaviors in the open field

Locomotor activity in the open field (Table 6 and Figure 14A) did not differ among groups as indicated by the same total number of crosses [$F(2, 29) = 1.60$, NS]. One-way ANOVA showed a significant effect of treatment [$F(2, 29) = 3.95$, $P < 0.05$]. The Duncan post hoc test showed that Ovx+E_2 rats spent more time in the inner zone and less time in outer zone of the open field when compared with other two groups ($P < 0.05$) (Table 6 and Figure 14B).

Table 5 The effects of estrogen on inhibitory avoidance and one-way escape tested in the elevated T-maze.

Withdrawal latency (seconds)	Ovx	Ovx+E_2	Pro
Baseline	22.33 ± 5.65	17.06 ± 4.09	26.11 ± 7.75
Avoidance 1	111.70 ± 29.50	53.35 ± 14.95	99.91 ± 29.13
Avoidance 2	154.25 ± 25.38^{b}	69.54 ± 13.63^{a}	157.53 ± 34.94^{b}
Escape	11.52 ± 1.61^{a}	14.04 ± 2.06^{a}	22.34 ± 2.71^{b}
Number of rats	11	11	11

Values are mean ± S.E.M. Different letters are significantly different from one other in the same parameters ($P < 0.05$).

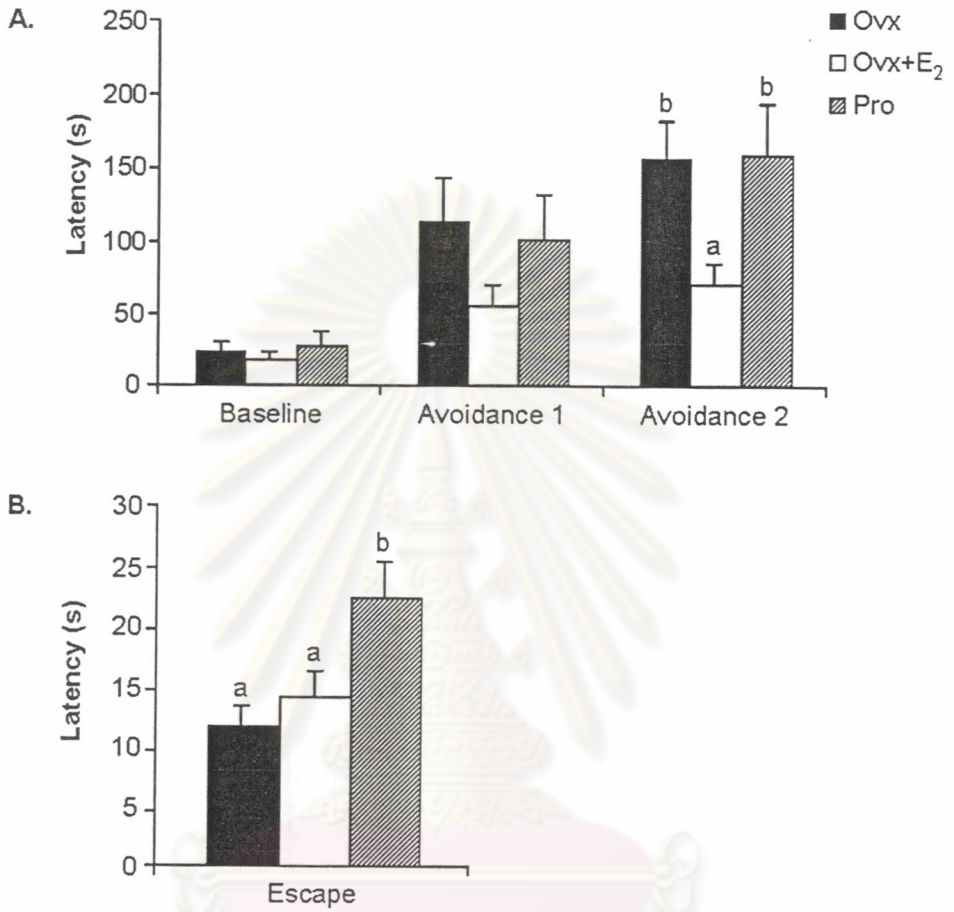

Figure 13. Histograms illustrate mean latency time (seconds ± S.E.M) of ovariectomized (Ovx), estrogen treated ovariectomized (Ovx+E_2), and proestrous (Pro) rats on (A) inhibitory avoidance task and (B) one-way escape task of the elevated T-maze. Number of rats was 11. Bars with different letters are significantly different from one another ($P < 0.05$).

Table 6 The effects of estrogen on locomotor activity and anxiety-liked behaviors tested in the open field.

Parameters	Ovx	Ovx+E_2	Pro
Total numbers of crosses (times)	98.82 ± 6.18	106.36 ± 6.42	94.91 ± 8.20
Time in outer zone (s)	222.80 ± 11.02^{b}	160.91 ± 18.06^{a}	221.73 ± 20.58^{b}
Time in inner zone (s)	77.20 ± 11.02^{b}	139.09 ± 18.06^{a}	78.27 ± 20.58^{b}
Number of rats	11	11	11

Values are mean ± S.E.M. Different letters are significantly different from one other in the same parameters ($P < 0.05$).

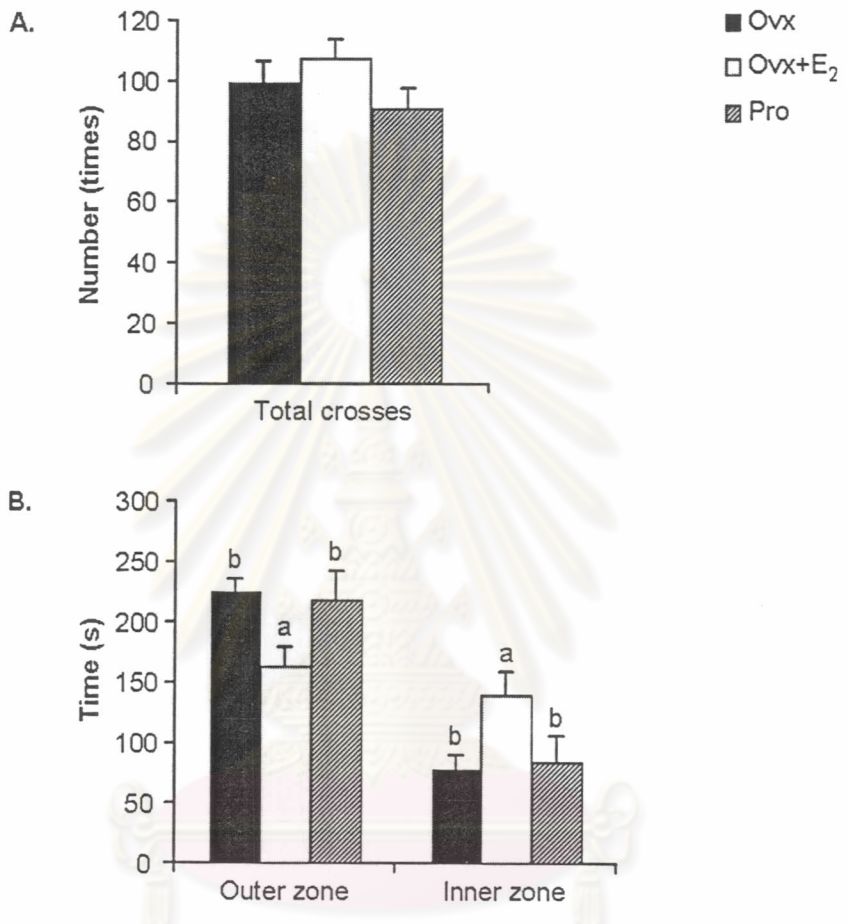

Figure 14. Histograms illustrate (A) mean numbers of total crosses (± S.E.M) and (B) mean time in seconds (± S.E.M) in outer and inner zone of ovariectomized (Ovx), estrogen treated ovariectomized (Ovx+E_2), and proestrous (Pro) rats in open field test. Number of rats was 11. Bars with different letters are significantly different from one another ($P < 0.05$).

2. The effects of estrogen on the serotonergic neurotransmission after the elevated T-maze test

After the ETM test, the rat's brains were immediately removed for measurement of 5-HT and 5-HIAA levels by HPLC technique. Figure 15 illustrated the examples of the chromatogram of 5-HT and 5-HIAA in hippocampus of Ovx (Figure 15A), Ovx+E_2 (Figure 15B), and Pro (Figure 15C) rats. Western blot analysis was used for measurement of SERT protein levels with in frontal cortex, nucleus accumbens, septum, amygdala and hippocampus, whereas TPH protein levels were measured in midbrain.

2.1. Serotonin and its metabolites levels in brain areas contributing to anxiety

Four weeks after ovariectomy, even though, 5-HT contents and its metabolite did not show significantly different in all groups (Table 7, Figures 16A, and 15B), the 5-HT turnover rate significantly increased in hippocampus [$F(2, 25) = 3.70$; $P < 0.05$] and nucleus accumbens [$F(2, 25) = 4.47$; $P < 0.05$] of Ovx+E_2 rats when compared to Ovx and Pro rats (Table 7 and Figure 16C).

There were, among the Ovx with or without estrogen administration, significant correlations between anxiety-liked behavior parameters of ETM test and 5-HT, its metabolite, and 5-HT turnover rate (Table 8). The significant positive correlations between the avoidance 2 latency times and 5-HT levels were found in amygdala [$r^2 = 0.43$; $F(1, 16) = 11.82$; $P < 0.01$] (Figure 17A), hippocampus [$r^2 = 0.41$; $F(1, 16) = 11.08$; $P < 0.01$] (Figure 18), and septum [$r^2 = 0.56$; $F(1, 16) = 20.70$; $P < 0.001$] (Figure 19A). There were negative correlations between the avoidance 2 latency times and 5-HT turnover rate in amygdala [$r^2 = 0.27$; $F(1, 16) = 5.78$; $P < 0.05$] (Figure 17B) and septum [$r^2 = 0.37$; $F(1, 16) = 9.41$; $P < 0.01$] (Figure 19C). In septum, it was also found a significant positive correlation between the avoidance 2 latency times and 5-HT's metabolite levels [$r^2 = 0.38$; $F(1, 16) = 9.65$; $P < 0.01$] (Figure 19B).

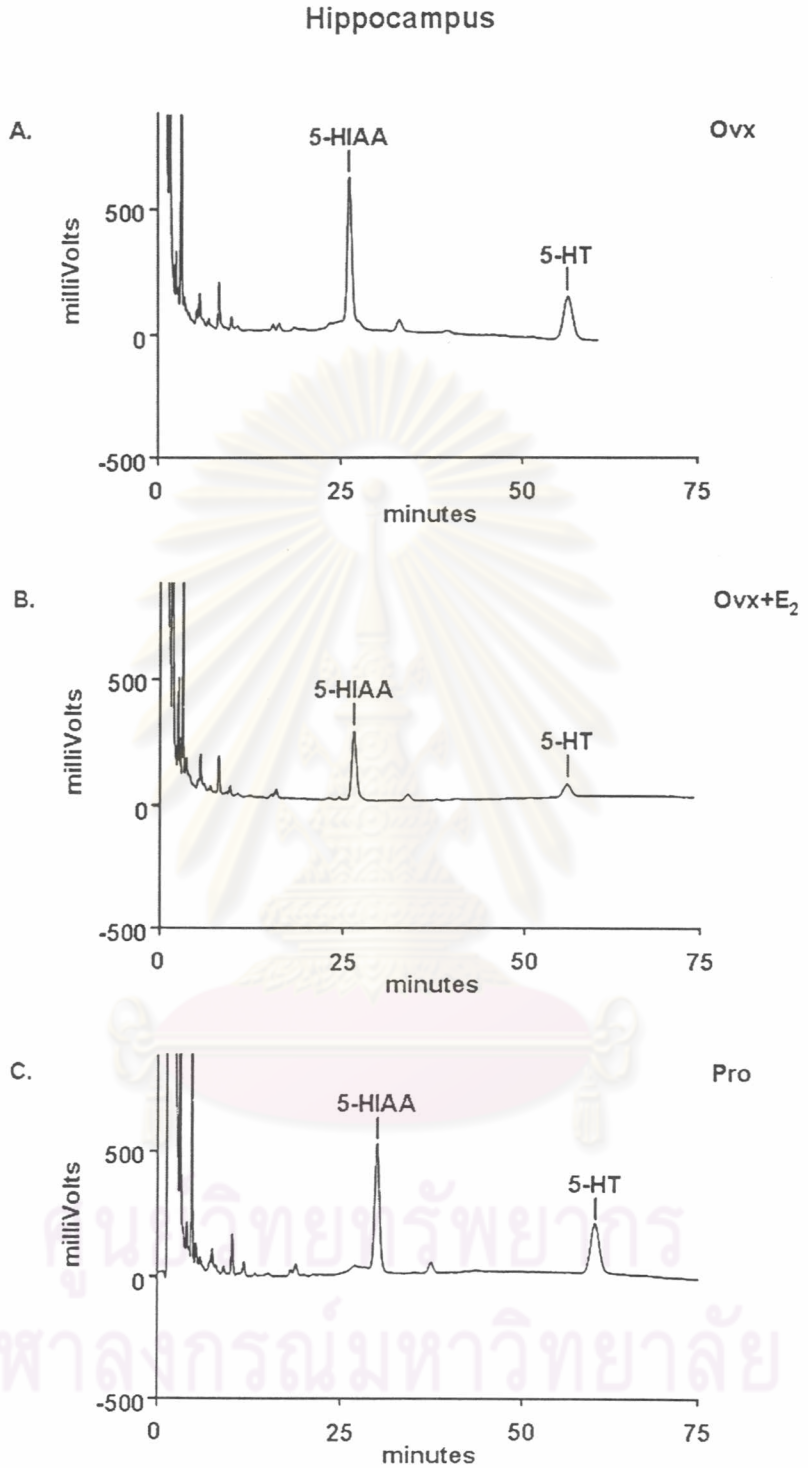

Figure 15. The chromatograms represent 5-HT and 5-HIAA levels in hippocampus of (A) ovariectomized (Ovx), (B) estrogen treated ovariectomized (Ovx+E_2), and (C) proestrous (Pro) rats measured by HPLC-EC. The retention times of 5-HIAA and 5-HT were approximately 29.6 and 59.5 minutes, respectively.

Table 7 The 5-HT, 5-HIAA levels, and 5-HIAA/5-HT ratio in frontal cortex (FC), amygdala (A), hippocampus (H), nucleus accumbens (NA), and septum (S) of ovariectomized (Ovx), estrogen treated ovariectomized (Ovx+E_2), and proestrous (Pro) rats.

	FC	A	H	NA	S
5-HT					
Ovx	3.92 ± 0.68	8.47 ± 1.14	6.94 ± 1.22	12.42 ± 1.29	8.88 ± 1.97
Ovx+E_2	2.76 ± 0.52	5.83 ± 0.78	4.07 ± 0.87	8.72 ± 2.05	4.87 ± 0.65
Pro	4.04 ± 0.57	8.07 ± 1.30	8.41 ± 2.42	10.84 ± 1.37	8.05 ± 1.19
5-HIAA					
Ovx	9.43 ± 0.81	14.13 ± 0.97	16.40 ± 1.30	19.64 ± 0.98	14.95 ± 1.07
Ovx+E_2	7.08 ± 0.67	11.53 ± 0.97	13.92 ± 1.71	19.97 ± 1.90	11.48 ± 0.92
Pro	10.18 ± 1.71	15.35 ± 2.96	17.17 ± 2.62	18.94 ± 1.59	16.87 ± 2.24
5-HIAA/5-HT					
Ovx	2.86 ± 0.32	1.85 ± 0.18	2.71 ± 0.26^{b}	1.73 ± 0.19^{b}	2.21 ± 0.32
Ovx+E_2	3.12 ± 0.46	2.17 ± 0.25	4.02 ± 0.57^{a}	3.04 ± 0.57^{a}	2.58 ± 0.28
Pro	2.54 ± 0.18	1.86 ± 0.11	2.71 ± 0.32^{b}	1.90 ± 0.17^{b}	2.21 ± 0.20

Values are mean ± S.E.M. Number of rats was 8-10. Different letters in the same column are significantly different from one another ($P < 0.05$).

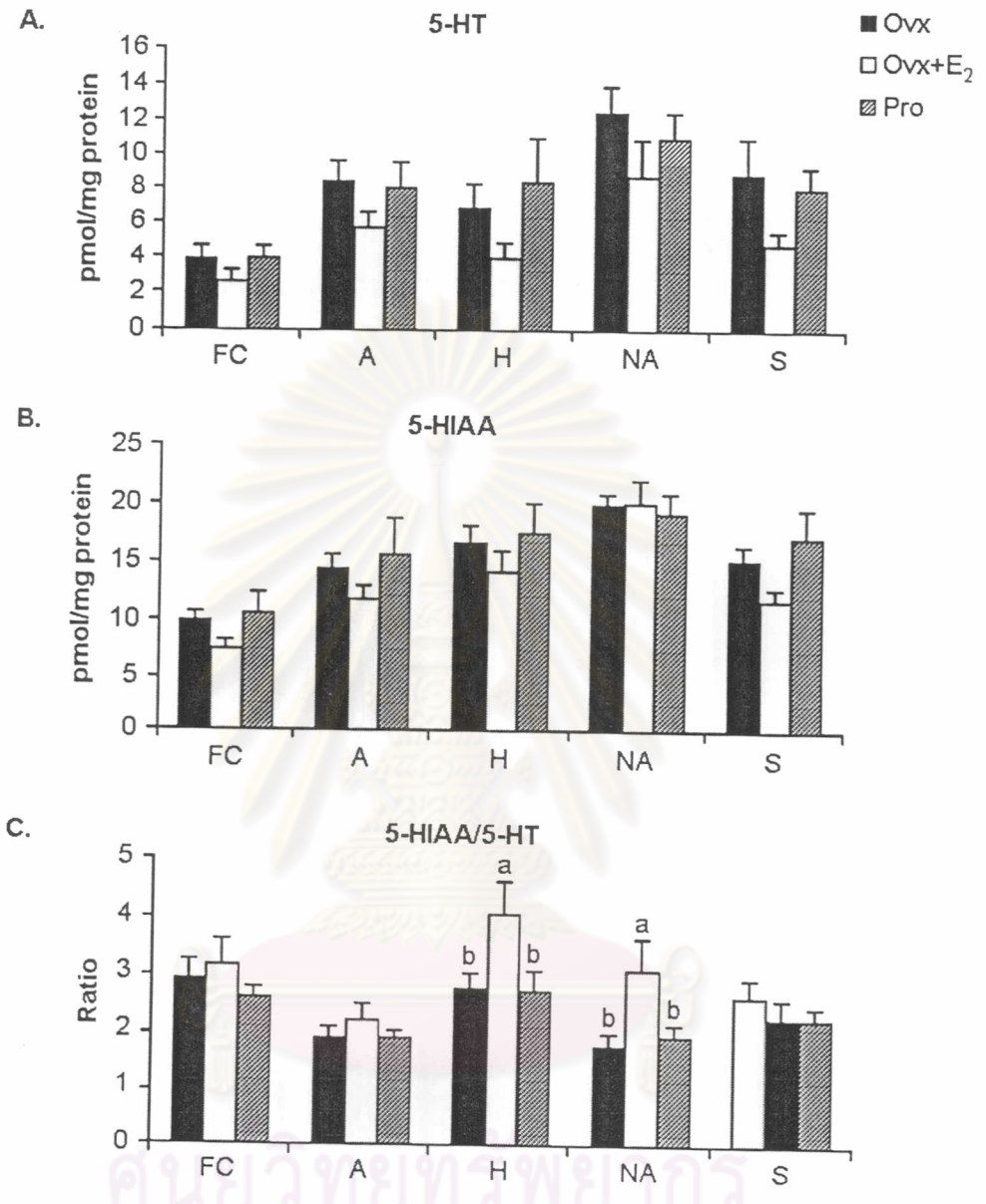

Figure 16. Histograms illustrate (A) mean (± S.E.M) serotonin contents, (B) 5-HIAA contents and (C) ratio of 5-HIAA/5-HT in frontal cortex (FC), amygdala (A), hippocampus (H), nucleus accumbens (NA), and septum (S) of ovariectomized (Ovx), estrogen treated ovariectomized (Ovx+E_2), and proestrous (Pro) rats. Number of rats was 8-10. Bars with different letters are significantly different from one another in the same brain region ($P < 0.05$).

Table 8 Correlation between the anxiety-liked behaviors tested with the elevated T-maze and 5-HT, 5-HIAA levels, and 5-HIAA/5-HT ratio in amygdala, hippocampus, and septum of ovariectomized and estrogen treated ovariectomized rats.

Brain areas	Factor 1	Factor 2	Correlation	*P*	r^2
Amygdala	Avoidance 2	5-HT	+	0.0034	0.4250
Amygdala	Avoidance 2	5-HIAA/5-HT	-	0.0287	0.2654
Hippocampus	Avoidance 2	5-HT	+	0.0043	0.4091
Septum	Avoidance 2	5-HT	+	0.0003	0.5641
Septum	Avoidance 2	5-HIAA	+	0.0068	0.3761
Septum	Avoidance 2	5-HIAA/5-HT	-	0.0074	0.3704

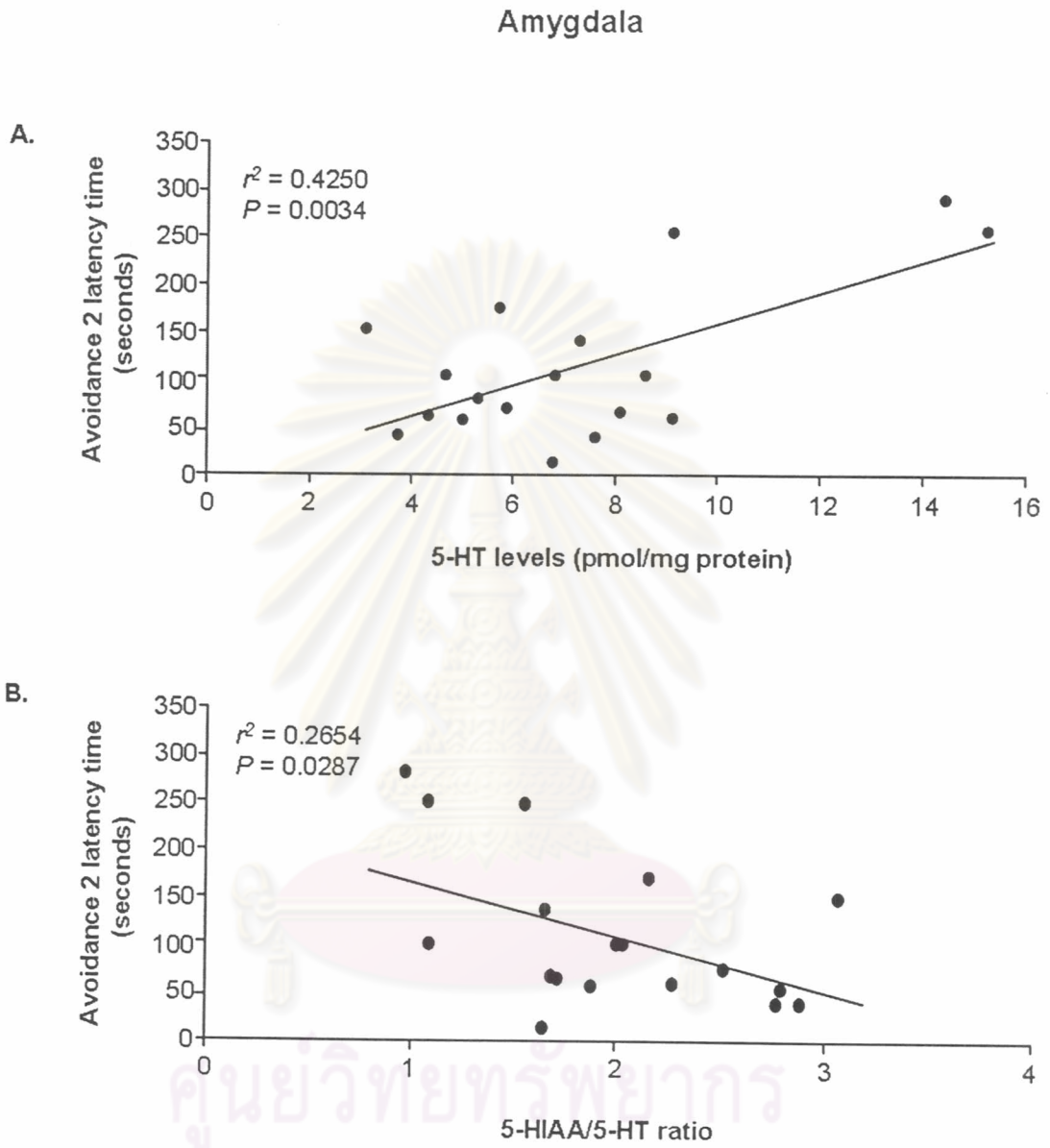

Figure 17. Regression analysis of (A) 5-HT levels and (B) 5-HIAA/5-HT ratio in the amygdala and the avoidance 2 latency time in the elevated T-maze test of ovariectomized and estrogen treated ovariectomized rats.

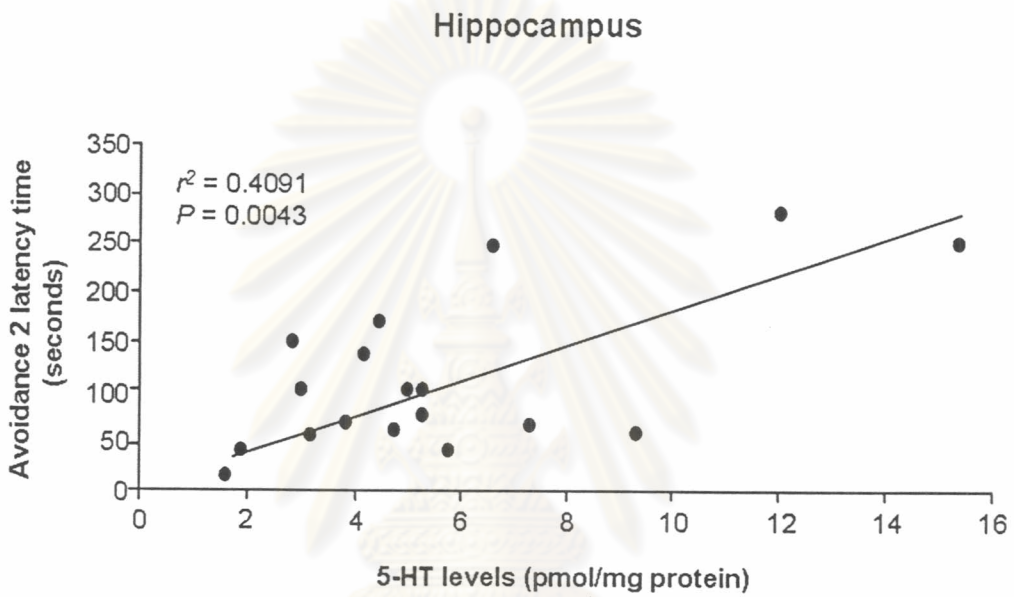

Figure 18. Regression analysis of 5-HT levels in the hippocampus and the avoidance 2 latency time in the elevated T-maze test of ovariectomized and estrogen treated ovariectomized rats.

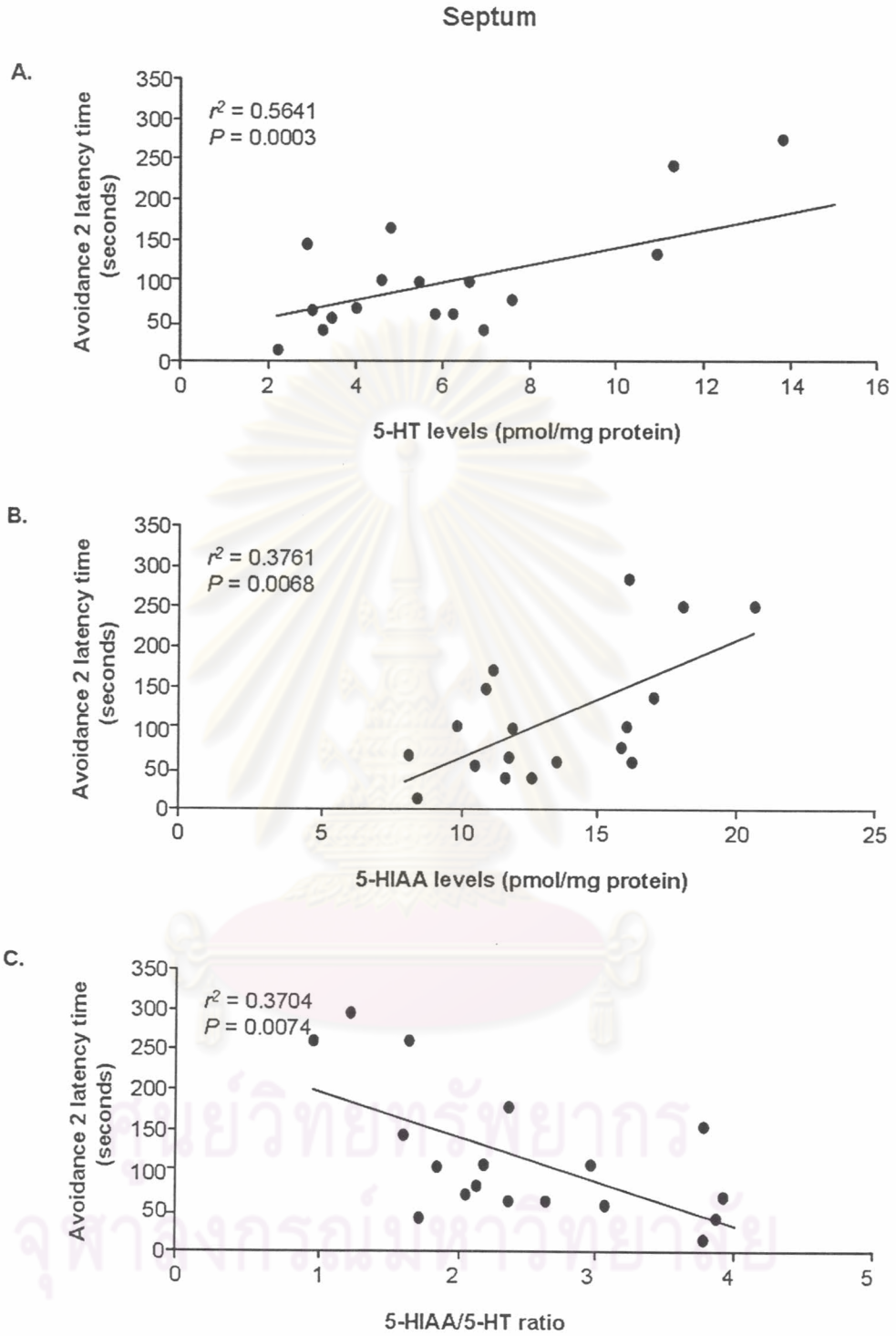

Figure 19. Regression analysis of (A) 5-HT levels and (B) 5-HIAA levels and (C) 5-HIAA/5-HT ratio in the septum and the avoidance 2 latency time in the elevated T-maze test of ovariectomized and estrogen treated ovariectomized rats.

2.2. TPH and SERT protein levels in brain areas contributing to anxiety

Figure 20A demonstrates an example of TPH and β-actin immunoreactive bands of Ovx, Ovx+E_2, and Pro rats in midbrain. Deprivation of ovarian sex hormones for 4 weeks significantly increased TPH protein levels [$F(2, 6) = 5.53$; $P < 0.05$] when compared to Ovx+E_2 and Pro rats (Figure 20B).

The SERT protein levels did not show any significantly different in all experimental groups and in all brain regions as shown in Figure 21.

3. The effects of estrogen on the postsynaptic serotonin receptor function on the anxiety-like behaviors tested with the ETM

In this experiment, three groups of rats including Ovx, Ovx+E_2, Pro rats were treated with ritanserin (5-$HT_{2A/2C}$ receptor antagonist) 30 minutes before behavioral tests.

3.1. 5-HT_2 antagonist effects on the anxiety-like behaviors in the ETM

Table 9 demonstrated that ritanserin at the dosage of 0.3 mg/kg significantly increased baseline latency time of Pro rats as compared with vehicle and other dosages [$F(3, 32) = 3.57$, $P < 0.05$]. Moreover, ritanserin at the dosage of 3.0 mg/kg significantly decreased both avoidance 1 [$F(3, 32) = 3.16$, $P < 0.05$] and avoidance 2 [$F(3, 32) = 4.30$, $P < 0.05$] latencies in Pro rats (Table 9) as compared to 0.3 and 1.0 mg/kg ritanserin, but no significantly different to vehicle treated rats. Finally, ritanserin at the dosage of 1.0 mg/kg significantly decreased in escape latency time of Pro rats as compared with vehicle and other dosages [$F(3, 32) = 3.59$, $P < 0.05$].

3.2. 5-HT_2 antagonist effects on the locomotor activity and anxiety-like behaviors in the open field

Locomotor activity in the open field (Table 10) did not differ among groups as indicated by the same total crosses. The Duncan post hoc test showed that 3.0 mg/kg ritanserin tended to increase time in the inner zone and decrease time in outer zone of Ovx rats [$F(3, 34) = 2.56$, $P = 0.0709$].

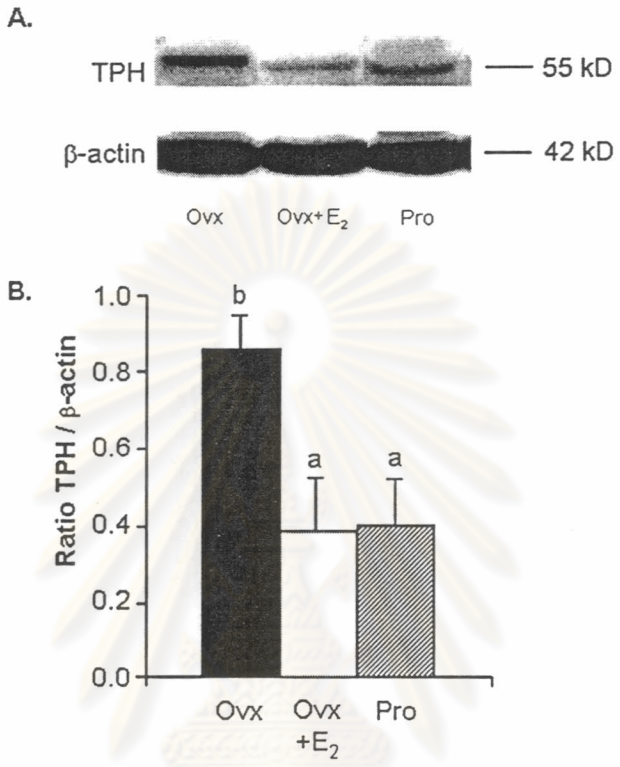

Figure 20. (A) Photograph represents the example of immunoreactive bands in Western blot probed with specific antibody to tryptophan hydroxylase enzyme (TPH) and β-actin. (B) Histogram illustrates mean (± S.E.M) of TPH/β-actin protein levels of ovariectomized (Ovx), estrogen treated ovariectomized (Ovx+E_2), and proestrous (Pro) rats in midbrain. Number of rats was 3. Bars with different letters are significantly different from one another ($P < 0.05$).

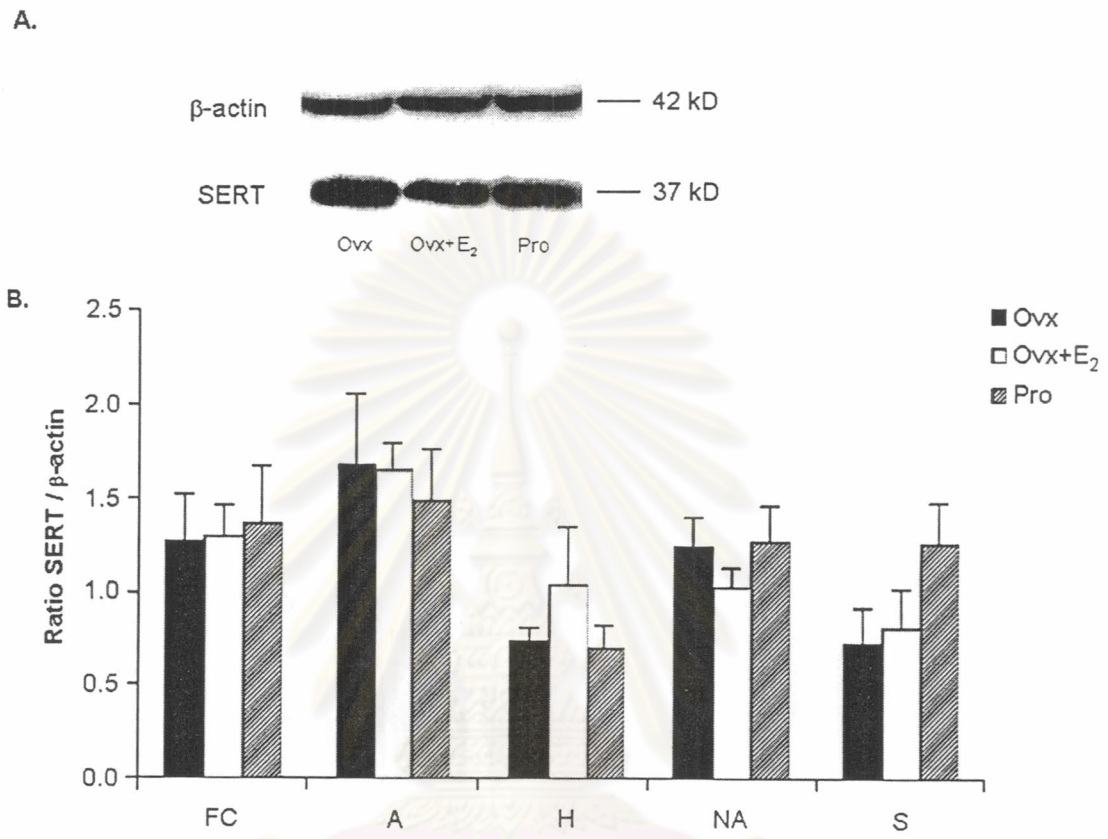

Figure 21. (A) Photograph represents the example of immunoreactive bands in Western blot probed with specific antibody to serotonin reuptake transporter (SERT) and β-actin in amygdala. (B) Histogram illustrates mean (± S.E.M) of SERT/β-actin protein levels in frontal cortex (FC), amygdala (A), hippocampus (H), nucleus accumbens (NA), and septum (S) of ovariectomized (Ovx), estrogen treated ovariectomized (Ovx+E_2), and proestrous (Pro) rats. Number of rats was 3-5.

Table 9 Effect of 5-$HT_{2A/2C}$ receptor antagonist (ritanserin) on inhibitory avoidance and one-way escape in the ETM

	Withdrawal Latency (mean ± S.E.M., s)				
	Inhibitory Avoidance			One-Way Escape	
Drug mg/kg	Baseline	Avoidance 1	Avoidance 2	Escape	*n*
Ovx+E_2					
0.0	17.06 ± 4.09	53.35 ± 14.95	69.54 ± 13.63	14.04 ± 2.06	11
0.3	15.18 ± 4.26	70.81 ± 20.98	92.00 ± 29.82	15.94 ± 4.80	6
1.0	21.02 ± 6.01	106.63 ± 18.54	133.61 ± 34.62	10.30 ± 1.70	10
3.0	14.80 ± 4.05	102.92 ± 28.83	130.94 ± 30.91	13.01 ± 2.45	9
Ovx					
0.0	22.33 ± 5.65	111.70 ± 29.50	154.25 ± 25.38	11.52 ± 1.61	11
0.3	36.28 ± 9.18	162.87 ± 36.68	162.49 ± 28.93	13.27 ± 3.24	8
1.0	19.13 ± 5.47	140.28 ± 36.44	178.91 ± 24.42	11.53 ± 2.69	10
3.0	20.07 ± 4.39	133.62 ± 30.95	207.40 ± 30.36	16.39 ± 2.75	9
Pro					
0.0	26.11 ± 7.75^{a}	$99.91 \pm 29.13^{a,b}$	$157.53 \pm 34.94^{a,b}$	22.34 ± 2.71^{a}	11
0.3	53.31 ± 13.47^{b}	172.01 ± 44.62^{a}	229.03 ± 41.43^{a}	$14.66 \pm 3.54^{a,b}$	8
1.0	21.80 ± 8.55^{a}	143.64 ± 34.27^{a}	240.10 ± 30.33^{a}	9.06 ± 2.50^{b}	8
3.0	14.02 ± 3.25^{a}	39.03 ± 16.95^{b}	87.17 ± 23.79^{b}	$14.95 \pm 3.05^{a,b}$	9

Different letters in the same column are significantly different from one another ($P < 0.05$).

Table 10 Effect of 5-$HT_{2A/2C}$ receptor antagonist (ritanserin) in open field test

Drug mg/kg	Total crosses	Time in outer zone (s)	Time in inner zone (s)	*n*
Ovx+E_2				
0.0	106.36 ± 6.42	160.91 ± 18.06	139.09 ± 18.06	11
0.3	129.50 ± 12.14	218.09 ± 14.23	81.92 ± 14.23	6
1.0	106.30 ± 9.48	222.77 ± 20.56	77.23 ± 20.56	10
3.0	105.44 ± 11.46	217.95 ± 20.39	82.05 ± 20.39	9
Ovx				
0.0	98.82 ± 6.18	222.80 ± 11.02[a]	77.20 ± 11.02[a]	11
0.3	105.25 ± 8.00	210.33 ± 13.75[a,b]	89.67 ± 13.75[a,b]	8
1.0	114.80 ± 6.60	229.28 ± 17.67[a]	70.73 ± 17.67[a]	10
3.0	116.22 ± 9.75	164.79 ± 27.15[b]	135.21 ± 27.15[b]	9
Pro				
0.0	94.91 ± 8.20	221.73 ± 20.58	78.27 ± 20.58	11
0.3	118.88 ± 7.11	217.80 ± 20.89	82.20 ± 20.89	8
1.0	102.00 ± 12.22	212.35 ± 15.08	87.65 ± 15.08	8
3.0	115.56 ± 8.91	183.47 ± 27.78	116.53 ± 27.78	9

Different letters in the same column are significantly different from one another ($P = 0.0709$).