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Anxiety-like behaviors and expression of SERT and TPH in the dorsal raphé of estrogen- and fluoxetine-treated ovariectomized rats

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ABSTRACT

The anxiolytic effect of fluoxetine (Flx) was often ineffective in postmenopausal and estrogen-deficient patients, but such effect had not been experimentally demonstrated, particularly in the female rat model of estrogen deficiency. Here we determined the anxiety-like behaviors in ovariectomized (Ovx) rats treated for 4 weeks with 10 μg/kg 17β-estradiol s.c. (Ovx + E2), 10 mg/kg Flx p.o. (Ovx + Flx) or a combination of both (Ovx + E2 + Flx). Since Flx is known to induce anxiolysis in males, we first evaluated the Flx regimen in male rats. The results showed that anxiety-like behaviors were reduced in Flx-treated male rats. In contrast, Ovx + Flx rats still exhibited the same anxiety-like behaviors as in Ovx rats. Both Ovx + E2 and Ovx + E2 + Flx rats, however, showed comparable reductions in anxiety-like behaviors, suggesting that Flx had no anxiolytic-like effect. Furthermore, E2 and E2 + Flx similarly upregulated the mRNA expression of serotonin reuptake transporter (SERT) and tryptophan hydroxylase-2 in the dorsal raphé of Ovx rats, while having no effect on SERT expression in the frontal cortex, hippocampus, septum, amygdala and periaqueductal gray. In conclusion, Flx induced anxiolytic-like action in male rats. In Ovx rats, it was E2 and not Flx that exerted the anxiolytic-like action, which was mediated, in part, by altering serotonin metabolism in the dorsal raphé.

1. Introduction

Several lines of evidence have suggested that estrogen modulated anxiety levels throughout a woman's life, while hypoestrogenic individuals run a greater risk of developing anxiety symptoms (Arpels, 1996). It was reported that anxiety disorders and mood disturbance, including depression and irritability, were common in postmenopausal patients (Bromberger et al., 2001; Colenda et al., 2010) and patients with bilateral oophorectomy (Rocca et al., 2008). Since estrogen normally regulates the number of serotonin receptors (e.g., 5-hydroxytryptamine [5-HT]$_{1A}$ receptor) and serotonin turnover in anxiety-related brain regions, such as hippocampus, amygdala and frontal cortex (Amin et al., 2005; Österlund et al., 2000), dysfunction of the brain's serotonergic system could be the cause of anxiety in postmenopausal patients. Thus, estrogen replacement therapy may be used to ameliorate anxiety symptoms in these patients (Strickler et al., 2000; von Mühlen et al., 1995).

Selective serotonin reuptake inhibitors (SSRIs), such as fluoxetine (Flx), citalopram and sertraline, the widely used serotonergic drugs, possess both antidepressive and anxiolytic actions (Kent et al., 1998; Vaswani et al., 2003). However, Flx was often ineffective as a remedy for depressive symptoms in postmenopausal and estrogen-deficient female patients (Pinto-Meza et al., 2006; Schneider et al., 1997), but its action was restored by estrogen supplementation (Westlund and Parry, 2003; Zanardi et al., 2007). These clinical findings appeared to suggest that the antidepressive action of Flx in women was estrogen-dependent. Moreover, the women's age, duration of estrogen deprivation and timing of estrogen replacement therapy might further affect the efficiency of Flx. Regarding the anxiolytic action, although Flx markedly mitigated anxiety disorders in young male and female patients (Fairbanks et al., 1997), whether its anxiolytic action was present in estrogen-deficient females has never been demonstrated experimentally.

Estradiol (E2) and Flx have been shown to induce anxiolysis by modulating the serotonergic system (for reviews see Bethea et al., 2002a; Kent et al., 1998). At the cellular level, E2 exerted the anxiolytic action via estrogen receptor-β (Lund et al., 2005; Tomihara et al., 2009). Both E2 (Chang and Chang, 1999) and SSRI (Apparsundaram et al., 2008) inhibited the serotonin reuptake transporter (SERT), thereby strengthening the serotonergic neurotransmission in the brain. Since brain serotonin levels could also be elevated by an increase in de novo serotonin biosynthesis, the E2- and/or Flx-induced upregulation of the rate-limiting enzyme, tryptophan hydroxylase (TPH) (Bethea et al., 2002b; Kim et al., 2002), was anticipated, particularly in the dorsal raphé, the principal site of serotonin production in the brain (Frazer and Hensler, 1999). However, the effects of E2 and Flx on SERT expression in estrogen-deficient animals

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remained elusive. It was possible that prolonged serotonin exposure in E2- or Flx-treated rats might induce an adaptive increase in SERT expression to enhance serotonin clearance.

The present study, therefore, aimed to investigate the effects of E2 and Flx independently or in combination on the anxiety-like behaviors in ovariectomized (Ovx) rats by using elevated plus-maze (EPM), elevated T-maze (ETM) and open field tests. Since several changes in anxiety-like behaviors, such as a decrease in the open arm time in the EPM test, were observed at 4 weeks after Ovx (Ho et al., 2007), the 4-week experimental period was used in this study. Moreover, SERT mRNA expression was determined in the major brain regions related to anxiety, namely the frontal cortex, hippocampus, septum, amygdala, periaqueductal gray and dorsal raphé, whereas the mRNA expressions of TPH1 and TPH2, the latter of which was predominant in the central nervous system (Sakowski et al., 2006), were investigated only in the dorsal raphé.

2. Materials and methods

2.1. Animals

Sexually mature male and female Wistar rats (*Rattus norvegicus*; 8 weeks old, weighing 180–210 g for males and 170–190 g for females) were obtained from the National Laboratory Animal Centre, Thailand. All animals were maintained in a husbandry unit at 25 ± 2 °C under 12-h light/dark cycle with lights on at 0600 h (average illuminance 200 lx), and fed standard chow and water ad libitum. Body weight was recorded daily. This study has been approved by the Animal Care and Use Committee of the Faculty of Medicine, Thammasat University, Thailand. The procedures are in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (publication no. 85-23, revised 1985).

2.2. Experimental design and treatment regimens

The experimental protocol for male rats is depicted in Fig. 1A. After acclimatization for 7 days, male rats were randomly divided into 2 groups, i.e., vehicle-treated (5 mL/kg distilled water p.o.; control) and Flx-treated groups (10 mg/kg Flx p.o.; Eli Lilly, IN, USA). Flx or vehicle was administered once daily (between 1300 and 1400 h) to non-sedated rats by gavage for 4 weeks prior to and during the 3 days of behavioral assessments.

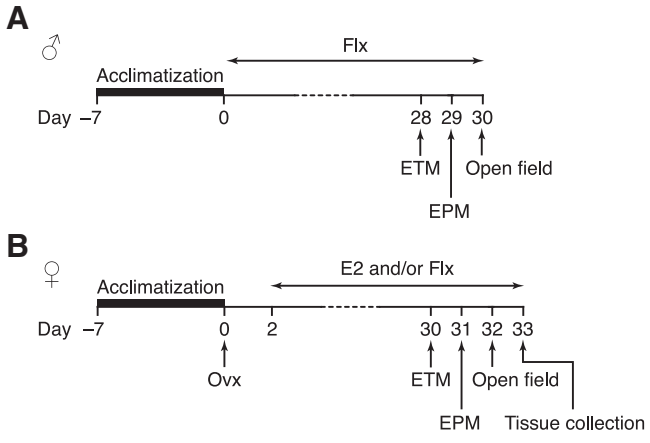

Fig. 1. Timelines show experimental protocols for male and female rats. (A) After 7-day acclimatization, male rats were daily administered for 4 weeks with distilled water (control) or 10 mg/kg Flx p.o. All rats were subjected to ETM, EPM and open field tests on days 28, 29 and 30, respectively. (B) In female rats, ovariectomy was performed on day 0. Thereafter, the animals were daily administered for 4 weeks with vehicle (Ovx), 10 μg/kg E2 s.c. (Ovx + E2), 10 mg/kg Flx p.o. (Ovx + Flx) or a combination of both (Ovx + E2 + Flx). All rats were subjected to ETM, EPM and open field tests on days 30, 31 and 32, respectively. At the end of the experiment, uteri and brains were collected for uterine weight and mRNA expression study, respectively.

Experimental protocol for female rats is shown in Fig. 1B. To induce E2 deficiency, bilateral ovariectomy was performed after 7 days of acclimatization under inhalational anesthesia with isoflurane (Minrad Inc., Bethlehem, PA, USA). Serum E2 levels in Ovx rats are low, usually less than 15 pg/mL, as compared to ~55 pg/mL in proestrous rats (Albert et al., 1991; Heaney et al., 2002). Thereafter, they were divided into four groups, i.e., Ovx group and Ovx groups treated with 10 μg/kg 17β-estradiol s.c. (Ovx + E2) (catalog no. E8875; Sigma, St. Louis, MO, USA), 10 mg/kg Flx p.o. (Ovx + Flx), or a combination of E2 and Flx (Ovx + E2 + Flx). The present Flx regimen has been reported to induce an anxiolytic-like effect in male rats (Zhang et al., 2000), while 10 μg/kg E2 was considered a physiological dose as it could induce behavioral effects similar to that observed in estrous rats (Diaz-Veliz et al., 1991). On each day, all rats were subjected to the same treatment, i.e., daily gavage (Flx or its vehicle) plus a subcutaneous injection (E2 or its vehicle). Vehicle treatments were 5 mL/kg distilled water p.o. and 0.25 mL/kg propylene glycol s.c. (Flx and E2 were dissolved in water and propylene glycol, respectively). E2, Flx or vehicles were administered once daily (between 1300 and 1400 h) for 4 weeks prior to and during the 3 days of behavioral assessments. Finally, uteri and brains were collected for uterine weight and mRNA expression study, respectively.

In all experiments, male and female rats were handled by the same researcher to minimize handling stress throughout the 7-day acclimatization and 4-week experimental periods. At the end of each experiment, the rats were subjected to three sequential behavioral assessments, i.e., ETM, EPM and open field tests (performed on different days between 0800 and 1100 h; Fig. 1). Each rat experienced each behavioral test only once without prior trials to avoid learning which could lead to inaccurate behavioral results. During each test, the animal was subjected to the test one at a time with no others in the same room.

2.3. Elevated plus-maze (EPM) test

The EPM apparatus was made of wood painted black. It was placed at a height of 50 cm, and consisted of two open arms (50 × 10 cm each) and two closed arms (50 × 10 cm with 30-cm walls), arranged so that the two pairs of identical arms were opposite to each other. During the behavioral test, the room had low noise levels with dim light of 20 lx (average illuminance in the husbandry unit during daytime is 200 lx). Each rat was placed in the apparatus for 5 min while being recorded by a high-definition infrared video camera (model HDR-XR 200E; Sony, Tokyo, Japan). The behavioral parameters obtained from the EPM test included time spent in open and closed arms, and the number of entries into these arms. An increase in the open arm activity provided an index of anxiolysis. The total number of arm entries and number of closed arm entries roughly indicated general activity of the rats, but such behaviors in the EPM test might be affected by various factors, such as trait anxiety and strain of animals (Hogg, 1996). The open field test was more suitable for evaluating locomotor activity (Schmitt and Hiemke, 1998).

2.4. Elevated T-maze (ETM) test

The ETM apparatus was also made of wood painted black. It consisted of three arms with equal dimensions (50 × 10 cm). One arm which was enclosed by walls (40-cm height) was perpendicular to the two opposing open arms. The apparatus was placed 50 cm above the floor. Average illuminance of the room during the test was 20 lx. Each test session consisted of three inhibitory avoidance trials with one escape trial held at 30 s intervals. In the first three inhibitory avoidance trials, each rat was placed at the distal end of the enclosed arm, facing the center of the maze. The baseline latency was defined as the time duration (in seconds) required for the rat to leave this arm

with all four paws. The same measurement was repeated in two subsequent trials (i.e., avoidance 1 and 2). Thereafter, the escape trial was performed by placing the animal at the end of the right open arm, facing the center of the maze. The time duration (in seconds) that the rat took to exit this arm with four paws was designated as the escape time. In general, the inhibitory avoidance represents conditioned fear, whereas the one-way escape from the open arm represents unconditioned fear (Graeff et al., 1993).

2.5. Open field test

The open field apparatus was a black wooden box (76×57 cm with 35-cm walls), marked with a 48-square grid (6×8 squares, 9.5 cm per side). Twenty-four peripheral squares were considered the outer zone, while the remaining 24 squares were the inner zone. Average illuminance of the room during the test was 20 lx. The total numbers of crosses that each rat made in a 5-min task represented the locomotor activity. Prolonged duration of time spent in the inner zone was indicative of anxiolytic-like behavior in rodents. However, the use of the open field test to investigate anxiety-like behavior in Flx-treated rats may not be suitable for the present study as this test was sensitive to the anxiolytic-like effects of benzodiazepines and 5-HT_{1A} receptor agonists, but not SSRIs (for review see Prut and Belzung, 2003). Therefore, in this study, we only used the open field test to evaluate locomotor activity.

2.6. Total RNA preparation, quantitative real-time PCR (qRT-PCR) and sequencing

After the behavioral assessments, animals were sacrificed and the frontal cortex, hippocampus, amygdala, septum, periaqueductal gray and dorsal raphé were dissected as described previously (Heffner et al., 1980). The qRT-PCR protocol used in this study was modified from the method of Charoenphandhu et al. (2009). In brief, the total RNA sample from each brain region was prepared by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Purity of the total RNA was determined by the ratio of spectrophotometric absorbance readings at 260 and 280 nm, which ranged between 1.8 and 2.0. Thereafter, 1 μg of total RNA was reverse-transcribed with iScript kit (Bio-rad, Hercules, CA, USA) by a thermal cycler (model MyCycler; Bio-rad). qRT-PCR and melting curve analyses for SERT, TPH1 and TPH2 expression were performed by the Bio-rad MiniOpticon real-time PCR system with the iQ SYBR Green SuperMix (Bio-rad) and specific primers (Table 1). Since E2 and Flx did not affect the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene (Supplementary Fig. S1), SERT and TPH expression levels were normalized by GAPDH expression. After each qRT-PCR study, PCR products were also visualized on 1.5% agarose gel stained with 1.0 μg/mL ethidium bromide under ultraviolet transilluminator (Alpha Innotech, San Leandro, CA, USA). After electrophoresis, PCR products were extracted by HiYield Gel/PCR DNA Extraction kit (Real Biotech Corporation, Taipei, Taiwan), and were sequenced by ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). qRT-PCR experiments were performed in triplicate.

Table 1
Rattus norvegicus oligonucleotide sequences used in qRT-PCR experiments.

Name	Accession no.	Primer (forward/reverse)	Product length (bp)
SERT	NM_013034	5′-CCACCTTCCCATACATTGT-3′ 5′-CTGTCTCCAAGAGTTTCTGC-3′	117
TPH1	NM_001100634	5′-GCTGAACAAACTCTACCCAAC-3′ 5′-TTCCCGATAGCCACAGTATT-3′	85
TPH2	NM_173839	5′-GGGTTACTTTCCTCCATCGGA-3′ 5′-AAGCAGGTTGTCTTCGGGTC-3′	86
GAPDH	NM_017008	5′-AGTCTACTGGCGTCTTCAC-3′ 5′-TCATATTTCTCGTGGTTCAC-3′	133

SERT, serotonin reuptake transporter; TPH1 and 2, tryptophan hydroxylase isoforms 1 and 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

2.7. Statistical analysis

Unless otherwise specified, the results from behavioral tests were expressed as means ± SE, while those from qRT-PCR were expressed as $\log_2$ means ± SE. Two-group comparisons were performed by Student's *t*-test. Multiple comparisons were analyzed by one-way analysis of variance (ANOVA) followed by Newman–Keuls post-test. Data from qRT-PCR were analyzed by the nonparametric Kruskal–Wallis test followed by Dunn's multiple comparison test. The level of significance was $p<0.05$. All statistical tests were performed by GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. The present Flx regimen effectively decreased the anxiety-like behaviors

Since anxiolytic-like actions of Flx have been studied in male rats (De Vry et al., 2004; Zhang et al., 2000), but not in E2-deficient female rats, prior to the investigation of Flx effect in Ovx rats, an experiment in male rats was performed to confirm the effectiveness of the present Flx regimen in reducing anxiety. At the start of Flx treatment, the rats in both control and Flx-treated groups had comparable body weight ($t=0.447$, $df=14$, $p>0.05$) (Fig. 2A). Thereafter, male rats treated with Flx for 4 weeks had lower body weight ($t=2.774$, $df=14$, $p<0.05$) and less daily weight gain ($t=2.830$, $df=14$, $p<0.05$) when compared to the vehicle-treated rats (control) (Fig. 2A). Both findings are typical responses consistent with previous reports (for review see Leibowitz and Alexander, 1998). Flx-treated male rats often entered ($t=2.927$, $df=14$, $p<0.05$) and spent more time ($t=2.187$, $df=14$, $p<0.05$) in the open arms of the EPM apparatus compared to the control rats, whereas the closed arm entry ($t=1.354$, $df=14$, $p>0.05$) and total entry ($t=0.118$, $df=14$, $p>0.05$) were not changed (Fig. 2B). In the ETM test, baseline avoidance times in control and Flx-treated male rats were not different ($t=0.376$, $df=14$, $p>0.05$). The inhibitory avoidance, which generally represented conditioned fear, was markedly impaired in Flx-treated male rats (avoidance 1: $t=3.711$, $df=14$, $p<0.05$; avoidance 2: $t=2.429$, $df=14$, $p<0.05$), whereas one-way escape from the open arm was not affected ($t=0.763$, $df=14$, $p>0.05$) (Fig. 2C). The Flx-treated and control male rats similarly spent more time in the outer zone of the open field arena than the inner zone. However, the outer zone time ($t=1.489$, $df=14$, $p>0.05$) and inner zone time ($t=0.167$, $df=14$, $p>0.05$) of Flx-treated male rats did not change when compared to those of control rats (Fig. 2D), indicating that the open field test, in contrast to EPM and ETM tests, could not reveal the anxiolytic effect of Flx, consistent to that suggested by Prut and Belzung (2003). Total numbers of crosses, which were indicative of locomotor activity, were comparable between the two groups ($t=0.210$, $df=14$, $p>0.05$) (Fig. 2D). The results thus indicated that the present Flx regimen was effective in alleviating anxiety in male rats, and did not lead to impairment of locomotion.

3.2. E2 supplementation abolished weight increase and uterine atrophy in Ovx rats

After acclimatization, the initial body weights of all female rats were comparable ($F_{3,43}=1.626$, $p>0.05$) (Fig. 3A). At the end of the 4-week experimental period, body weight ($F_{3,43}=50.621$, $p<0.05$) and daily weight gain ($F_{3,43}=35.514$, $p<0.05$) of Ovx rats were significantly greater than those of Ovx + E2 rats (Fig. 3A and B). Body weight and daily weight gain of Ovx + Flx rats were slightly but significantly less than those of Ovx rats, while Ovx + E2 + Flx rats showed a further reduction in weight gain (Fig. 3A and B). In addition, E2 supplementation restored the uterine weight ($F_{3,43}=155.612$, $p<0.05$) in both Ovx and Ovx + Flx rats (Fig. 3C).

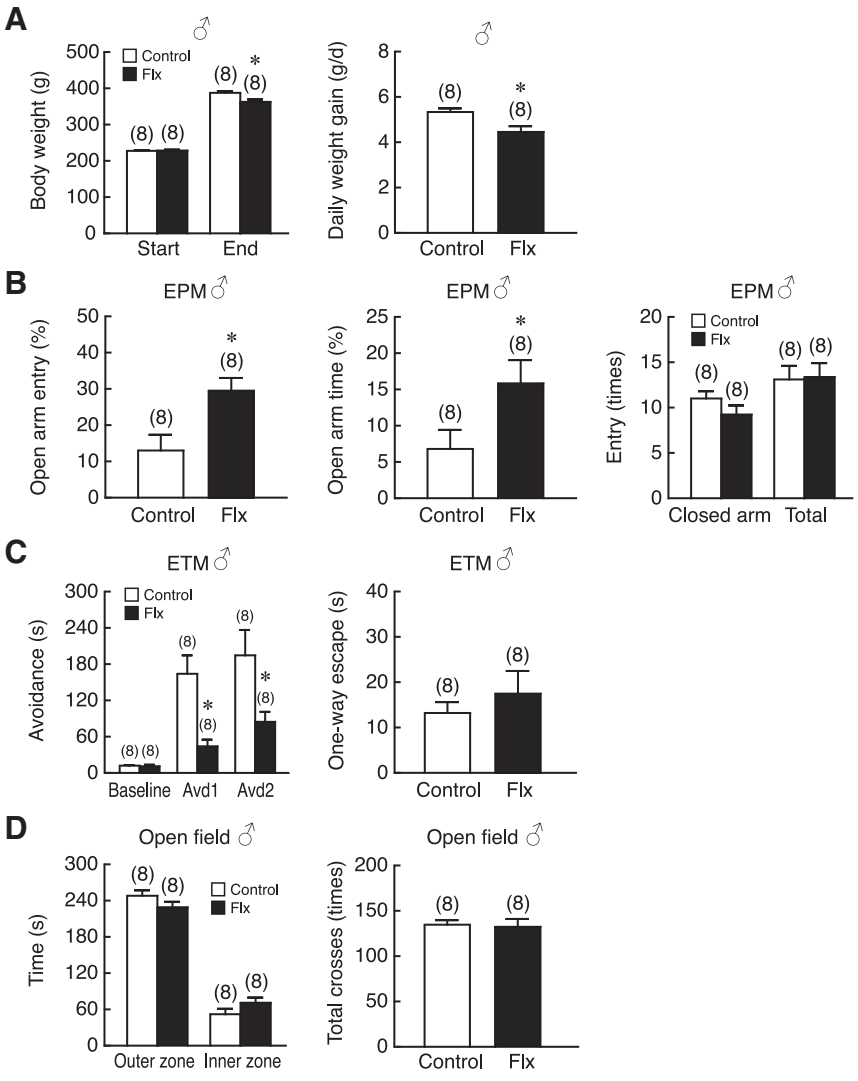

Fig. 2. (A) Body weight and daily weight gain, and (B–D) anxiety-related parameters of male rats orally administered for 4 weeks with distilled water (control) or 10 mg/kg Flx. Body weight was measured at the start and the end of 4-week treatment period. All rats were subjected to EPM, ETM and open field tests. Total entry of EPM test was the sum of open and closed arm entries. Avd1 and Avd2 denote avoidance 1 and 2, respectively. Numbers in parentheses represent the numbers of experimental animals. * $p<0.05$, compared with its respective control group.

3.3. E2 but not Flx had anxiolytic-like effect in Ovx rats

The EPM results showed that Ovx + E2 rats spent more time in the open arms ($F_{3,43}=5.424$, $p<0.05$), and had a greater number of entries into the open arms ($F_{3,43}=6.255$, $p<0.05$) than Ovx rats, indicating an anxiolytic-like effect of E2 in rats (Fig. 4A and B). Interestingly, in contrast to male rats, Flx administration did not reduce the anxiety-like behaviors in Ovx + Flx rats since Ovx + Flx rats showed no changes in open arm entry and open arm time when compared to Ovx rats (Fig. 4A and B). However, combined administration of E2 and Flx showed an anxiolytic-like effect, but did not exhibit an additive effect in Ovx + E2 + Flx rats (Fig. 4A and B), suggesting that Flx probably had no anxiolytic-like action in E2-supplemented Ovx rats. Neither E2 nor Flx altered the closed arm entries ($F_{3,43}=1.626$, $p>0.05$), whereas the total entry ($F_{3,43}=3.571$, $p<0.05$) was increased in Ovx + E2 rats (Fig. 4C).

The results from the ETM test were consistent with those from the EPM test since Ovx + E2 and Ovx + E2 + Flx, but not Ovx + Flx rats, manifested a decrease in anxiety-like behaviors when compared with Ovx rats, as indicated by the impaired inhibitory avoidance (avoidance 1: $F_{3,43}=4.409$, $p<0.05$; avoidance 2: $F_{3,43}=3.574$, $p<0.05$) (Fig. 5A). The baseline avoidance times of Ovx, Ovx + E2 and Ovx + E2 + Flx rats were lower than that of Ovx + Flx rats ($F_{3,43}=4.104$, $p<0.05$) (Fig. 5A), suggesting that Flx decreased exploratory activity in Ovx + Flx rats. There was no significant difference in the escape trial ($F_{3,43}=0.126$, $p>0.05$) (Fig. 5B). Taken together, the results indicated that only E2, but not Flx, could induce anxiolysis in E2-deficient female rats.

3.4. Both E2 and Flx had no effect on locomotion in Ovx rats

There was no significant difference in the time spent in both outer ($F_{3,43}=0.186$, $p>0.05$) and inner zones ($F_{3,43}=0.149$, $p>0.05$) of the open field arena (Fig. 6A). The total numbers of crosses in the open field were comparable among the four groups of animals ($F_{3,43}=0.638$, $p>0.05$) (Fig. 6B), indicating that the locomotor activity was not impaired by either E2 or Flx.

3.5. E2 and Flx upregulated SERT and TPH2 mRNA expression in the dorsal raphé

Since serotonin is an important modulator of anxiety in both humans and rats (Eison and Eison, 1994), we investigated SERT mRNA expression in various brain regions related to the anxiety-like behaviors, i.e., frontal cortex, hippocampus, septum, amygdala, periaqueductal

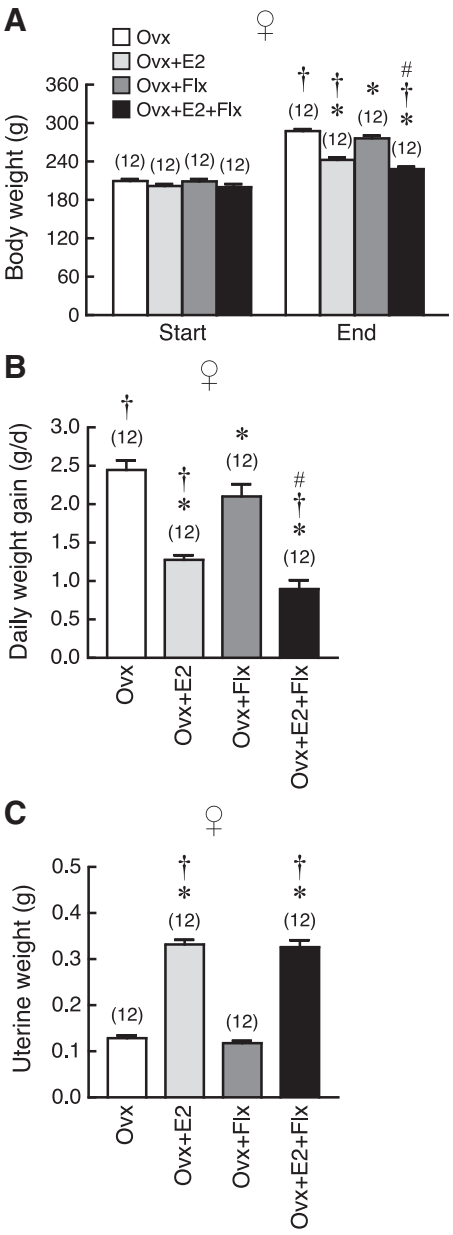

Fig. 3. (A) Body weight gain, (B) daily weight gain, and (C) uterine weight of ovariectomized rats daily administered for 4 weeks with vehicles (Ovx), 10 μg/kg E2 s.c. (Ovx + E2), 10 mg/kg Flx p.o. (Ovx + Flx) or a combination of both (Ovx + E2 + Flx). Vehicle treatments were 5 mL/kg distilled water p.o. (for Ovx and Ovx + E2 groups) and 0.25 mL/kg propylene glycol s.c. (for Ovx and Ovx + Flx groups). Body weight was measured at the start and the end of the 4-week treatment period, whereas uterine weight was determined at the end of the 4-week treatment period. Numbers in parentheses represent the numbers of experimental animals. * $p<0.05$, compared with Ovx group; † $p<0.05$, compared with Ovx + Flx group; # $p<0.05$, compared with Ovx + E2 group.

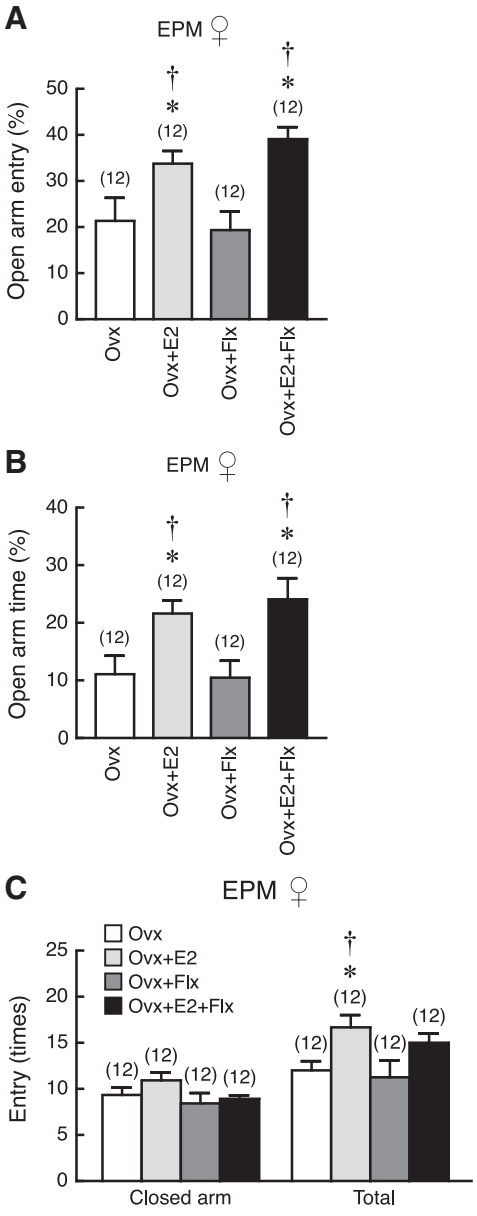

Fig. 4. (A) Open arm entry, (B) open arm time, and (C) closed arm and total entries as determined by EPM test in ovariectomized rats daily administered for 4 weeks with vehicles (Ovx), 10 μg/kg E2 s.c. (Ovx + E2), 10 mg/kg Flx p.o. (Ovx + Flx) or a combination of both (Ovx + E2 + Flx). Vehicle treatments were 5 mL/kg distilled water p.o. (for Ovx and Ovx + E2 groups) and 0.25 mL/kg propylene glycol s.c. (for Ovx and Ovx + Flx groups). Total entry is the sum of both open and closed arm entries. Numbers in parentheses represent the numbers of experimental animals. * $p<0.05$, compared with Ovx group; † $p<0.05$, compared with Ovx + Flx group.

gray and dorsal raphé. TPH mRNA expression was determined only in the dorsal raphé, which is the major site of serotonin synthesis (Frazer and Hensler, 1999). With the use of qRT-PCR, in all experimental groups, SERT mRNA expression was more abundant in the periaqueductal gray and dorsal raphé than in other brain regions (Fig. 7A). In the dorsal raphé, TPH2 was more abundant than TPH1 (Fig. 7B). Amplicon sequencing confirmed the correct SERT and TPH sequences.

E2 supplementation to Ovx rats significantly upregulated SERT and TPH2 mRNA expression in the dorsal raphé by 2.39- and 4.06-fold, respectively (Fig. 7A and B), and E2 + Flx administration upregulated SERT and TPH2 by 6.77- and 12.52-fold, respectively. However, for both transcripts, such expression levels in Ovx + E2 and Ovx + E2 + Flx groups were not statistically different. On the other hand, the mRNA expressions of SERT and TPH2 in the dorsal raphé of Ovx + Flx rats were not altered when compared to those of Ovx rats (Fig. 7A and B). Neither E2 nor Flx affected TPH1 mRNA expression in the dorsal raphé or SERT mRNA expression in other brain regions (Fig. 7A and B). These results collectively suggested that E2 alleviated anxiety, in part, by altering biosynthesis and metabolism of serotonin.

4. Discussion

The present study provided corroborative evidence that Flx was probably without anxiolytic-like effects in both E2-deficient (Ovx + Flx) and E2-repleted (Ovx + E2 + Flx) female rats, as evaluated by the

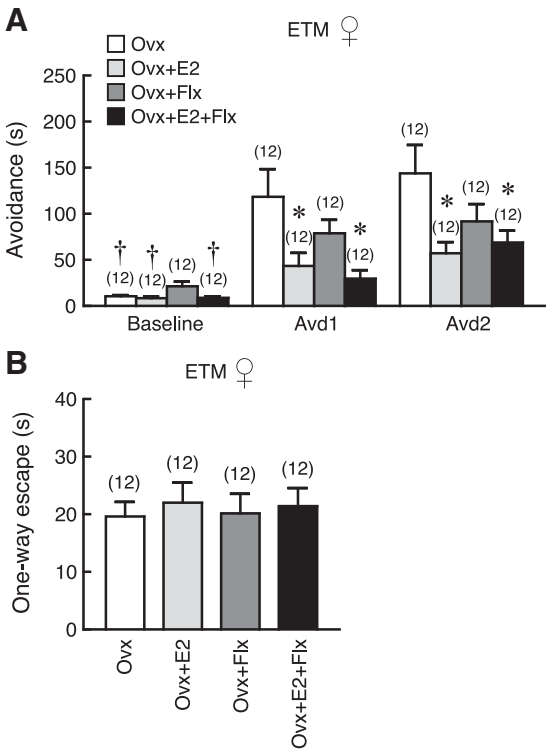

Fig. 5. (A) Avoidance latency, and (B) one-way escape latency as determined by ETM test in ovariectomized rats daily administered for 4 weeks with vehicles (Ovx), 10 μg/kg E2 s.c. (Ovx + E2), 10 mg/kg Flx p.o. (Ovx + Flx) or a combination of both (Ovx + E2 + Flx). Vehicle treatments were 5 mL/kg distilled water p.o. (for Ovx and Ovx + E2 groups) and 0.25 mL/kg propylene glycol s.c. (for Ovx and Ovx + Flx groups). Avd1 and Avd2 denote avoidance 1 and 2, respectively. Numbers in parentheses represent the numbers of experimental animals. * $p<0.05$, compared with Ovx group; † $p<0.05$, compared with Ovx + Flx group.

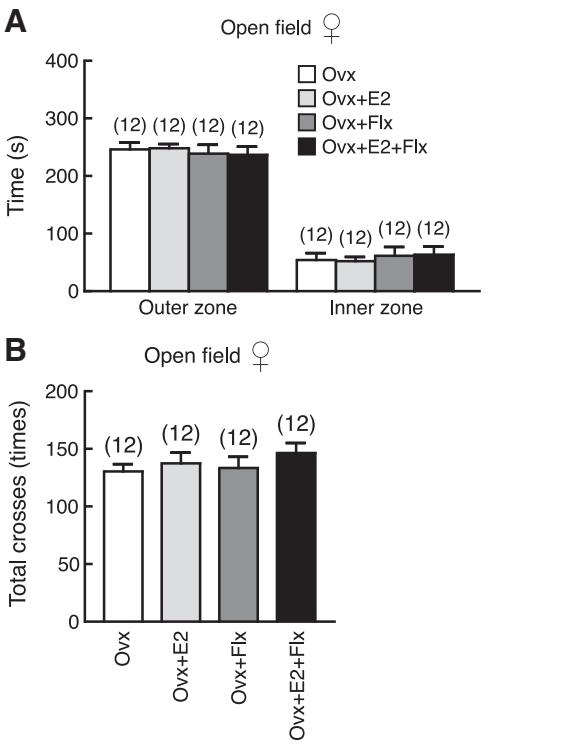

Fig. 6. (A) Time spent in the outer and inner zones, and (B) total number of crosses in the open field arena as determined in ovariectomized rats daily administered for 4 weeks with vehicles (Ovx), 10 μg/kg E2 s.c. (Ovx + E2), 10 mg/kg Flx p.o. (Ovx + Flx) or a combination of both (Ovx + E2 + Flx). Vehicle treatments were 5 mL/kg distilled water p.o. (for Ovx and Ovx + E2 groups) and 0.25 mL/kg propylene glycol s.c. (for Ovx and Ovx + Flx groups). Numbers in parentheses represent the numbers of experimental animals.

EPM and ETM tests. In contrast, its anxiolytic-like action was clearly demonstrated in male rats (Fig. 2B), confirming the previous reports (De Vry et al., 2004; Zhang et al., 2000). In addition, since the impaired inhibitory avoidance in Ovx rats was clearly seen after E2 supplementation, the anxiolytic-like action of E2 may result from mitigation of conditioned or learned fear, an anxiety-like behavior which implied generalized anxiety disorder in humans (Graeff et al., 1993; 1996). On the other hand, no change in the one-way escape trial indicated that E2 did not affect unconditioned or innate fear, an anxiety-like behavior similar to panic disorder in humans (Graeff et al., 1993). Such E2 and Flx effects were not due to impairment of locomotor function since there was no change in the numbers of crosses in the open field test. The anxiolytic-like actions of E2 in Ovx rats may be explained by the upregulation of SERT and TPH2 in the dorsal raphé, which is the major site of serotonin production (Frazer and Hensler, 1999). Despite having no effect on anxiety-like behaviors, Flx did affect female rats in an E2-dependent manner since a reduction in body weight gain was apparent in Ovx + E2 + Flx rats when compared with Ovx + E2 rats, presumably due to the Flx-induced decrease in food intake resulting from alteration of serotonergic activation in the medial hypothalamic nuclei (Leibowitz and Alexander, 1998).

Until now, there has been no animal-based evidence on the anxiolytic-like effects of SSRI in female rats although the estrogen-dependent effect of SSRI was previously demonstrated in the rodent paradigms of depressive disorders (Estrada-Camarena et al., 2004; Sell et al., 2008). Specifically, a combined E2 and SSRI treatment, but not E2 or SSRI alone, produced an antidepressant-like effect in rats as indicated by the reduced immobility and the increased swimming behaviors in the forced swimming test (Estrada-Camarena et al., 2004; Sell et al., 2008). Moreover, an unreliable outcome of SSRI used as antidepressants in estrogen-deficient patients has been reported in a number of clinical studies (Pae et al., 2009; Pinto-Meza et al., 2006; Schneider et al., 1997). For instance, Schneider et al. (1997) reported that Flx administration without estrogen replacement therapy in postmenopausal women showed minimal benefit in the treatment of major depressive disorder. On the other hand, estrogen supplementation to estrogen-deficient patients was found to restore the antidepressive effects of SSRI (Nagata et al., 2005; Schneider et al., 2001; Westlund and Parry, 2003; Zanardi et al., 2007), and a combined estrogen and SSRI administration was more effective than estrogen alone in the treatment of depressive symptoms and hot flashes in oophorectomized women (Nagata et al., 2005). In contrast to the antidepressive action, SSRI such as Flx appeared to have no anxiolytic-like action in estrogen-deficient rats with or without E2 supplementation.

The enhancement of serotonergic neurotransmission in specific brain nuclei, such as amygdala, is generally the key mechanism of anxiolysis in both humans and rodents (Inoue et al., 2004). Thus, the elevation of synaptic serotonin levels that resulted from the inhibition of SERT activities was used to explain the anxiolytic action of SSRI (Izumi et al., 2006). Nevertheless, the present results suggested that such a mechanism was gender-specific since Flx could produce anxiolytic-like action in male rats with relatively low plasma E2 levels. Although the effect of Flx in male rats was partially dependent on male sex steroids (Fink et al., 1999), testosterone may not be the active hormone required for the anxiolytic-like action of Flx in male rats. Martínez-Mota et al. (2008) reported a reduction in the antidepressive-like action of Flx in adult male rats treated with aromatase inhibitor, suggesting that it was E2 locally produced in the brain from testosterone, but not testosterone itself, which modulated the Flx action.

However, unlike SSRI, E2 itself could induce anxiolysis by various mechanisms. One study reported that the numbers of serotonergic receptors in several brain regions, such as frontal cortices, striatum

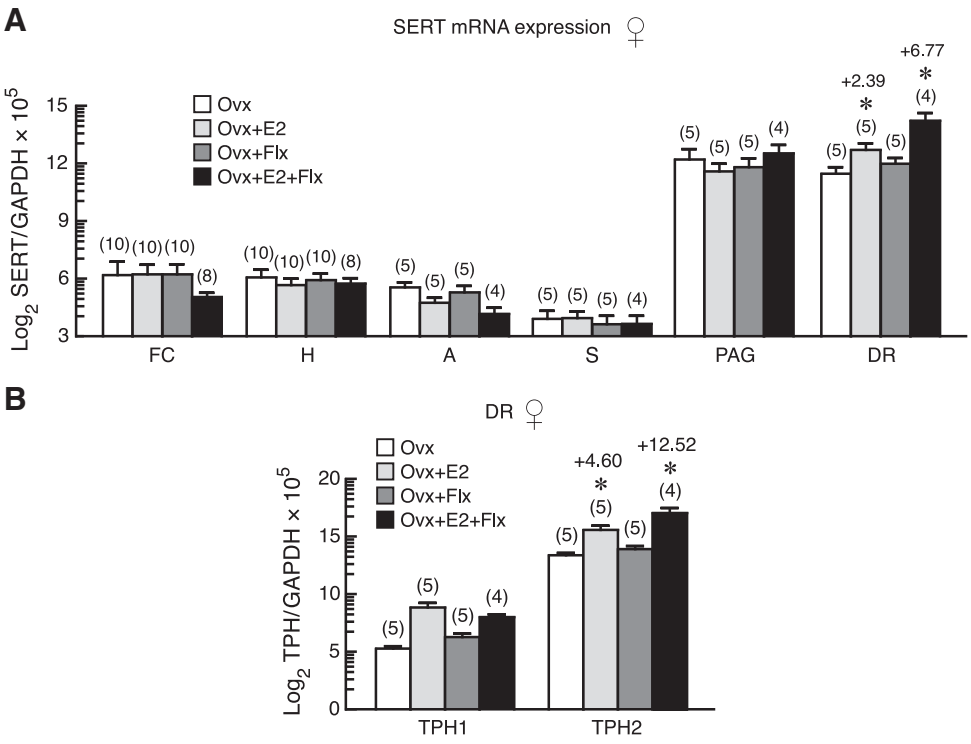

Fig. 7. (A) SERT mRNA expression in various brain regions, including frontal cortex (FC), hippocampus (H), amygdala (A), septum (S), periaqueductal gray (PAG) and dorsal raphé (DR), dissected from ovariectomized rats daily administered for 4 weeks with vehicles (Ovx), 10 μg/kg E2 s.c. (Ovx + E2), 10 mg/kg Flx p.o. (Ovx + Flx) or a combination of both (Ovx + E2 + Flx). Vehicle treatments were 5 mL/kg distilled water p.o. (for Ovx and Ovx + E2 groups) and 0.25 mL/kg propylene glycol s.c. (for Ovx and Ovx + Flx groups). (B) Expression of TPH1 and TPH2 mRNA in the dorsal raphé. Expression levels were determined by qRT-PCR, and normalized by GAPDH expression. Fold change values are presented along with their respective statistical marks. Plus sign (+) in front of the fold change value means upregulation of gene. Numbers in parentheses represent the numbers of independent samples. * $p<0.05$, compared with Ovx group (Kruskal–Wallis test).

and nucleus accumbens, were significantly decreased in Ovx rats, and E2 supplement restored the receptor density, presumably by upregulating serotonergic receptor expression (Cyr et al., 2000). Several in vitro studies also suggested the E2-induced inhibition of SERT activity, which led to synaptic serotonin accumulation similar to the action of SSRI (Chang and Chang, 1999; Koldzic-Zivanovic et al., 2004). The elevated synaptic serotonin levels could also be achieved by the E2-induced desensitization of presynaptic 5-HT_{1A} and 5-HT_{1B} receptors (Hiroi and Neumaier, 2009; Raap et al., 2000), which normally inhibit serotonin release. In addition, serotonin production could be increased by E2-induced upregulation of TPH2 mRNA expression in the dorsal raphé (Fig. 7B), as previously reported in rodents (Hiroi et al., 2006) and non-human primates (Bethea et al., 2002b). However, this adaptive response appeared to be site-specific as it was not observed in the whole midbrain preparation (Pandaranandaka et al., 2009). Even in the dorsal raphé, upregulation of TPH2 mRNA in the caudal subregion, but not the rostral dorsomedial subregion, was found to be associated with lower anxiety-like behavior (Hiroi et al., 2006). Besides the upregulation of TPH2 by E2, an increase in synaptic serotonin concentration may be accomplished by the E2-induced inhibition of monoamine oxidase type A, which normally degrades serotonin and norepinephrine (Holschneider et al., 1998; Ma et al., 1995). Taken together, the anxiolytic-like effect of E2 in Ovx rats was likely to result from increases in postsynaptic serotonergic receptor density and synaptic serotonin concentrations (i.e., SSRI-like effects) in specific brain regions.

Long-term exposure to high concentrations of synaptic serotonin induced a compensatory upregulation of SERT mRNA in the dorsal raphé of E2-treated Ovx rats (Fig. 7A). In contrast, a 4-h direct exposure to serotonin resulted in SERT protein internalization in cultured serotonergic neurons (Kittler et al., 2010). This SERT overexpression might, in turn, alter serotonin turnover in the remote brain regions innervated by the raphé fibers. Our finding was thus consistent with a previous report that E2 injection, which mimicked E2 positive feedback for gonadotropin surge, increased the density of SERT in several brain regions, e.g., septum, basolateral amygdala and ventromedial hypothalamic nucleus (McQueen et al., 1997). However, due to the controversial findings regarding SERT expression, it was not known whether the upregulation of SERT was merely a compensatory response to enhance serotonin turnover, or was the essential underlying mechanism for the anxiolytic-like actions of E2 in Ovx rats.

The absence of change in SERT and TPH2 mRNA expression in Ovx + Flx rats was consistent with the findings from behavioral studies. This finding suggested that Flx had no anxiolytic-like effects in Ovx rats, even after E2 supplementation, but the role of other ovarian steroids, particularly progesterone (Bethea et al., 2002a), could not be excluded. Alternatively, in the presence of E2, Flx might have an anxiolytic-like action that shared the same mechanism with E2, which had already produced maximal behavioral responses.

In conclusion, we provided supportive evidence that E2 was essentially an anxiolytic-like agent in estrogen-deficient female rats, whereas Flx was without such action in Ovx or Ovx + E2 rats. Our results thus agreed with the clinical studies that suggested the use of estrogen replacement therapy in postmenopausal women (Schneider et al., 1997; Westlund and Parry, 2003; Zanardi et al., 2007). Furthermore, at the cellular level, E2 may partially exert its anxiolytic-like effects by modulating serotonergic metabolism, such as upregulation of TPH and SERT for the enhancement of serotonin production and turnover, respectively.

Supplementary materials related to this article can be found online at doi: 10.1016/j.pbb.2011.02.023.

Conflict of interest

The authors declare no conflicts of interest.

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