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ERβ-Selective Estrogen Receptor Modulators Produce Antianxiety Behavior when Administered Systemically to Ovariectomized Rats

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17β-Estradiol (E_2) may influence anxiety behavior; however, its effects and mechanisms are not well understood. To determine whether E_2's effects on anxiety behavior may involve actions at intracellular estrogen receptor (ER) α or β isoforms, selective ER modulators (SERMs) were administered (10 μg; s.c.) to ovariectomized rats 48 h before testing for anxiety behavior. Rats received sesame oil vehicle, 17β-E_2, which has a high affinity for ERα and ERβ, or SERMs that vary in their activity at ERα and β. ERα-selective SERMs were propyl pyrazole triol (PPT), which has more selective effects at ERα, than does the other ERα SERM utilized, 17α-E_2, which also binds ERβ. ERβ-selective SERMs were diarylpropionitrile (DPN) and 7,12-dihydrocoumestan (coumestrol). DPN is more selective at ERβ than coumestrol, which also binds ERα. 17β-E_2 and ERβ-selective SERMs (DPN, coumestrol) produced clear antianxiety behavior in the open field, elevated plus maze, emergence, light–dark transition, defensive freezing, and Vogel punished drinking tasks. Anxiety behavior of rats administered ERα-selective SERMs (PPT, 17α-E_2) was not different from vehicle; however, PPT and 17α-E_2 enhanced sexual receptivity in a manner similar to 17β-E_2. Coadministration of tamoxifen (10 mg/kg) blocked the antianxiety behavior produced by 17β-E_2, DPN, or coumestrol. Together, these data suggest that actions at ERβ may underlie some of E_2's antianxiety effects.
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INTRODUCTION

Estradiol (E_2) may influence the incidence and/or expression of anxiety among women. Generalized anxiety disorder occurs in ~5% of the general population; however, the incidence increases to 10% for women aged 40 and older (Wittchen and Hoyer, 2001), a group that has E_2 levels that are on the decline. Reports of anxiety are also increased during other periods of relatively low E_2 (ie premenstrually, postpartum): whereas, when E_2 levels are greater, either naturally or via hormone therapy (HT), women's reports of anxiety decrease (Arpels, 1996; Campbell and Whitehead, 1977; Halbreich, 1997; Torizuka *et al*, 2000). Some women with premenstrual syndrome treated with transdermal patches of E_2 report decreased anxiety (Smith *et al*, 1995); however, opposite effects are also reported (Schmidt *et al*, 1998). Together, these findings underscore the importance of investigating the role, substrates, and mechanisms associated with E_2's effects on anxiety behavior.

E_2 also has antianxiety effects in animal models. On proestrus, when E_2 levels peak, rats spend more time on the open arms of the elevated plus maze, more time in social interaction with a conspecific, and less time freezing in response to shock than do females in other phases of the estrous cycle or male rats (Fernandez-Guasti *et al*, 1999; Frye *et al*, 2000; Mora *et al*, 1996). Ovariectomy (ovx) typically increases (Diaz-Veliz *et al*, 1997; Mora *et al*, 1996; Morgan and Pfaff, 2001; Nomikos and Spyraki, 1988), and E_2 replacement decreases (Nomikos and Spyraki, 1988; Frye and Walf, 2004, 2005; Walf and Frye, 2005), anxiety behavior of rodents. For example, ovx rats or mice administered systemic or intrahippocampal E_2 spend more time on the open arms of the elevated plus maze than do their vehicle-administered counterparts (Nomikos and Spyraki, 1988; Frye and Walf, 2004, 2005; McCarthy *et al*, 1996; Walf and Frye, 2005). In the open field task, E_2 has been reported to produce anxiolytic, anxiogenic, or no effects (Morgan and Pfaff, 2001; Frye and Walf, 2004; Leret *et al*, 1994; Walf and Frye, 2005). Indeed, the nature of E_2's effects on anxiety behavior seems to depend upon the regimen, and its effects on activity and stress responsiveness (Walf and Frye, 2005).

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There are many possible mechanisms by which E_2 can influence anxiety behavior. E_2 can act through traditional intracellular E_2 receptors (ERs), and bind to the E_2 response element (ERE), or the AP-1 binding site. Although E_2 may also have actions independent of ERs, E_2's actions at ERs may influence anxiety behavior. Intact female ERβ knockout mice spent less time on the open arms of the elevated plus maze compared to wild type and ERα knockout mice (Krezel *et al*, 2001). Similarly, ovx, E_2-replaced ERβ knockout mice of another strain demonstrated greater anxiety behavior than did their wild-type counterparts in the plus maze (Imwalle *et al*, 2005). Together, these data support further investigation of E_2's actions via ERs for its effects on anxiety behavior.

Variable effects of E_2 on anxiety behavior may be related to its actions at the two distinct ERs. Two ER subtypes, ERα and ERβ, have been discovered and are localized in different areas of the brain. There is ERα mRNA in the ventromedial hypothalamic nucleus and subfornical organ. ERβ mRNA seems to be more widely distributed across many regions (olfactory nuclei, zona incerta, ventral tegmental area, cerebellum, laminae III–V, VII, and IX of the spinal cord, pineal gland). Other brain regions containing both ERα and ERβ mRNA include the bed nucleus of the stria terminalis, medial and cortical amygdaloid nuclei, preoptic area, lateral habenula, periaqueductal gray, parabrachial nucleus, locus ceruleus, nucleus of the solitary tract, spinal trigeminal nucleus, and superficial laminae of the spinal cord. As well, both forms of ER mRNA are localized to the cerebral cortex and hippocampus; however, the hybridization signal in these areas is much weaker for ERα than ERβ mRNA (Shughrue *et al*, 1997). The differential distribution of the two ER subtypes leaves open the possibility that ERα and ERβ may have different behavioral functions.

To begin to dissociate the extent to which actions at ERα and/or ERβ mediate estrogens' effects on anxiety behavior, we have begun to examine functional effects of selective ER modulators (SERMs). As there has been very little systematic investigation of behavioral effects of SERMs, we based our hypothesis on evidence that ERα has an essential role in reproduction (Hewitt and Korach, 2003), the signal for ERβ seems to be stronger than the signal for ERα in the hippocampus, an important brain area for E_2's modulation of anxiety behavior (Frye *et al*, 2000; Frye and Walf, 2002, 2005; Shughrue *et al*, 1997), and recent findings which suggests that ERβ-selective SERMs are more effective than ERα-selective SERMs or vehicle to modulate affective behavior of ovx female rats (Lund *et al*, 2005; Walf *et al*, 2004). Thus, we hypothesized that if actions at ERβ mediate antianxiety behavior, then SERMs with more selective activity at ERβ would produce greater antianxiety behavior than would SERMs with more selective activity at ERα or vehicle.

MATERIALS AND METHODS

These methods were preapproved by the Institutional Animal Care and Use Committee at SUNY Albany.

Animals and Housing

Female Long-Evans rats ($N = 240$), approximately 55 days old, were obtained from our breeding colony at SUNY-Albany (original stock from Taconic Farms, Germantown, NY). Rats were group housed (4–5 per cage) in polycarbonate cages ($45 \times 24 \times 21\,cm^3$) in a temperature-controlled room ($21 \pm 1^{\circ}C$) in The Laboratory Animal Care Facility. The rats were maintained on a 12/12 h reversed light cycle (lights off 0800) with continuous access to Purina Rat Chow and tap water. All rats were ovx under Rompun (12 mg/kg; Bayer Corp., Shawnee Mission, KS) and Ketaset (60 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA) anesthesia 1 week prior to testing.

SERMs

Rats were administered sesame oil vehicle, 17β-E_2 (Steraloids, Newport, RI), which has equal affinity for ERα and ERβ (Kuiper *et al*, 1997), or one of the following SERMs, as per our previously published methods (Walf *et al*, 2004).

ERα-specific. SERMs: Propyl pyrazole triol (PPT; Tocris Cookson, Inc., Ellisville, MO) is a potent selective ER agonist that has 410-fold selectivity for ERα over ERβ (Stauffer *et al*, 2000). 17α-E_2 (Sigma Chemical Co., St Louis, MO) is five times more active at ERα than ERβ (Kupier *et al*, 1997).

ERβ-specific SERMs: Diarylpropionitrile (DPN; Tocris Cookson, Inc., Ellisville, MO) is a highly selective ERβ agonist, with 70 times greater activity at ERβ than ERα (Meyers *et al*, 2001). 7,12-dihydrocoumestan (coumestrol; Sigma Chemical Co., St Louis, MO) is a less selective ERβ agonist, with a sevsen-fold greater affinity for ERβ compared to ERα (Kuiper *et al*, 1997, 1998).

Dosing

Ovx rats received 10 μg SERMs or vehicle s.c. and were tested 48 h later. This regimen was based upon our previous investigation of dose-dependent effects of E_2 on anxiety behavior. Compared to lower or higher dosages, 5–10 μg 17β-E_2 s.c. to young, ovx rats is most effective at producing physiological plasma levels of E_2 and antianxiety behavior, 48 h later (Walf and Frye, 2005). As well, this E_2 regimen is commonly employed to induce sexual receptivity in rats (Frye *et al*, 1998). Although in some cases, higher concentrations of DPN and/or PPT are necessary to induce the same biological activity as E_2 (Handa *et al*, 1986, 1987), 10 μg concentrations were utilized for all SERMs, to minimize loss of selective actions at ERs, and/or specific behavioral effects, that might be produced by supraphysiological and/or chronic SERM regimen. We have also used this regimen of SERM-administration successfully to examine E_2's modulation of depressive behavior of ovx rats in the forced swim test (Walf *et al*, 2004).

ER Blockade

Some rats were coadministered tamoxifen (10 mg/kg s.c.), or vehicle, in conjunction with behaviorally effective SERMs and were tested, 48 h later. Tamoxifen was utilized because it is a nonselective, but effective, ER antagonist that readily penetrates the blood–brain barrier. This tamoxifen regimen blocks lordosis facilitated by s.c. E_2 (Etgen and Shamamian, 1986).

Procedure

Experiment 1. To determine effects of ERα- and ERβ-selective SERMs for sexual receptivity, ovx rats ($n = 10$/grp) were randomly assigned to receive oil vehicle or 10 μg 17β-E_2, PPT, 17α-E_2, DPN, or coumestrol 48 h before testing for sexual receptivity with a stimulus male and motor behavior in the horizontal crossing task.

Experiment 2. To determine effects of ERα- and ERβ-selective SERMs for anxiety behavior, ovx rats ($n = 10$/grp) were randomly assigned to receive oil vehicle or 10 μg 17β-E_2, PPT, 17α-E_2, DPN, or coumestrol 48 h before anxiety testing.

Experiment 3. To determine whether effects of ERβ-selective SERMs for anxiety behavior can be attenuated with coadministration of an ER antagonist, ovx rats ($n = 10$/grp) were randomly assigned to receive vehicle or tamoxifen (10 mg/kg s.c.), followed by 10 μg of 17β-E_2, DPN, or coumestrol. Rats were then tested 48 h later for anxiety behavior.

Behavioral Testing

Rats in Experiment 1 were tested for sexual receptivity, whereas rats in Experiments 2 and 3 were tested for anxiety behavior in the following tasks. In Experiments 2 and 3, some rats were sequentially tested once a week, for up to 4 weeks, until performance in each task was examined. Other rats were only tested in a single task. No rats were ever tested more than once in any given task because of known test-decay effects in most measures of anxiety behavior.

Sexual receptivity. Rats were tested for sexual behavior in a Plexiglas chamber ($50 \times 25 \times 30\ cm^3$) with an intact male for 10 mounts or 10 min, whichever occurred first, by an observer who was blind to rats' experimental conditions. The frequency of lordosis (lordosis quotient) and the intensity of lordosis (lordosis ratings; LR), quantified by rating dorsiflexion during lordosis on a scale of 0–3, exhibited by experimental female rats were recorded (Frye *et al*, 1998; Hardy and DeBold, 1971).

Horizontal crossing task. Immediately after testing for sexual receptivity, rats were tested in the horizontal crossing task as per Frye *et al* (2000). Rats were placed in a $39 \times 39 \times 30\ cm^3$ Digiscan Optical Animal Activity Monitor (Accuscan Instruments Inc., Columbus, OH) that mechanically recorded the number of beam breaks that occurred during a 5-min period.

Open field. The open field task was used in accordance with previously published methods (Frye *et al*, 2000; McCarthy *et al*, 1995). The open field ($76 \times 57 \times 35\ cm^3$) has a 48-square grid floor (6×8 squares, 9.5 cm/side), and an overhead light illuminating the central squares (all but the 24 perimeter squares are considered central). The number of central and peripheral squares (summed for total) entered during a 5-min period were recorded.

Elevated plus-maze. The methods previously described by Frye *et al* (2000) were utilized. The elevated plus-maze consisted of four arms, 49 cm long and 10 cm wide, elevated 50 cm off the ground. Two arms are enclosed by walls 30 cm high and the other two arms are exposed. Rats are placed at the junction of the open and closed arms. The number of entries, and the amount of time spent, on the open and closed arms during a 5-min period were recorded.

Emergence test. As previously described (Frye *et al*, 2000), rats were placed in a closed opaque cylinder ($21 \times 7 \times 7\ cm^3$) that was set in an open field and secured to prevent rolling. The lid of the cylinder was removed and the latency for the rat to emerge completely from the cylinder was recorded (maximum latency 5 min).

Light–dark transition task. Rats were placed on the side of a two-chambered box ($30 \times 40 \times 40\ cm^3$) with white walls and floor and illuminated by a 40-W light from above; the other side of the box was painted black and had a lid so it was not illuminated. For 5-min, the time spent on the light side of this chamber compared to the dark side was recorded (Chaouloff *et al*, 1997).

Defensive freezing. The defensive freezing procedure utilized was according to methods previously reported (Frye *et al*, 2000). Briefly, rats were placed in a clear Plexiglas chamber ($26.0 \times 21.2 \times 24.7\ cm^3$). In the right rear corner was a pedestal (2.5 cm diameter, 10.0 cm height), which was wrapped by wires connected to a shock source (Lafayette Model A615B, Lafayette, IN) set to deliver 6.66 mA of unscrambled shock, initiated by the experimenter and terminated by the rats' withdrawal of its paws. The response to footshock was recorded by the experimenter as a flinch-jump rating (1 = flinch, 2 = jump, 3 = jump and squeak). The latency to touch the shock prod, and the time spent freezing in response to shock, was recorded for 15 min.

Vogel punished drinking task. After 24 h of water deprivation, rats were placed in a clear plexiglas chamber with a metal grid floor ($44 \times 22 \times 20\ cm^3$; Brocco *et al*, 1990). An electrified water bottle was suspended from the ceiling of the chamber and connected to a computer interface (Anxio-meter, Columbus Instruments, Colombus, OH) that automatically recorded the number of licks and shocks (one shock for every 20 licks) that the rat received during the 3-min test. The test began after the rat made an initial 20 licks and received its first shock (0.3 mA for 2 s). Rats had a maximum latency of 15 min to begin the test. Data were excluded from rats that did not fulfill this criteria ($n = 4$).

Statistical Analyses

In Experiments 1 and 2, one-way analyses of variance (ANOVAs) were utilized to determine if there were differences among SERMs' effects on behavior. In Experiment 3, two-way ANOVAs were utilized to determine if there were differences among SERMs' and tamoxifen's effects on behavior. The α level for statistical significance

was $p \leqslant 0.05$ and a trend was considered $p \leqslant 0.10$. Where appropriate, *post hoc* tests used to determine group differences were Fisher's tests with Boneferroni corrections.

RESULTS

Experiment 1: Effects of SERMs for Sexual Receptivity and Horizontal Crossing

There were effects of 17β-E_2 and ERα-selective SERMs to enhance sexual receptivity compared to vehicle or ERβ-selective SERMs. There were differences among groups in the lordosis quotients of rats in response to mounting by a stimulus male ($F_{5,54} = 4.86$; $p < 0.01$). *Post hoc* tests revealed that administration of 17β-E_2, PPT, or 17α-E_2 significantly increased lordosis quotients compared to vehicle, DPN, or coumestrol (see Figure 1).

There were no significant differences among groups of rats administered vehicle (704 ± 66), 17β-E_2 (720 ± 128), PPT (788 ± 97), 17α-E_2 (943 ± 80), DPN (867 ± 111), or coumestrol (826 ± 77) in the number of beam breaks made in the horizontal crossing task ($p = 0.49$).

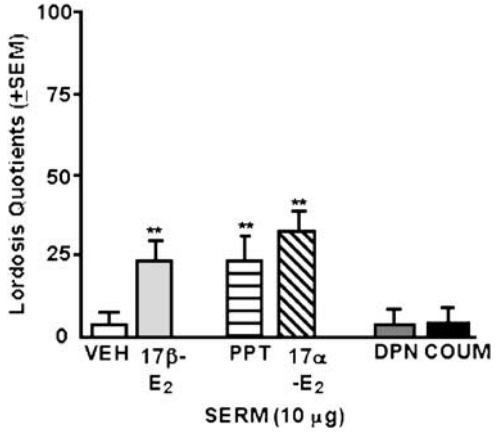

Figure 1 The mean ($\pm$SEM) lordosis quotients of ovx rats administered vehicle, 17β-E_2, PPT, 17α-E_2, DPN, coumestrol (COUM) 48 h before testing ($n = 10$/condition). ** above bar indicates a significant difference from vehicle, DPN, and coumestrol ($p < 0.05$).

Experiments 2 and 3: Effects of SERMS and/or Tamoxifen on Anxiety Behavior

Open field. 17β-E_2 and ERβ-selective SERMs produced antianxiety effects in the open field task compared to vehicle or ERα-selective SERMs. There were differences among groups in the number of central ($F_{5,54} = 3.19$; $p < 0.01$; see Figure 2a) and total entries ($F_{5,54} = 2.69$; $p < 0.03$; see Table 1) made in the brightly-lit open field. *Post hoc* tests revealed that 17β-E_2, DPN, or coumestrol (which all bind ERβ), significantly increased the number of central entries made compared to rats administered vehicle. 17β-E_2 significantly increased the number of central entries compared to PPT or 17α-E_2.

As was observed for the first group of rats tested in Experiment 2, 17β-E_2 and ERβ-selective SERMs had antianxiety effects in the open field task in Experiment 3. Significant differences among groups in the number of central entries ($F_{3,72} = 4.67$; $p < 0.01$; Figure 2b) were due to 17β-E_2, DPN or coumestrol having more central entries compared to vehicle-administered rats. There was a tendency for differences among groups for peripheral entries ($F_{3,72} = 2.12$; $p < 0.10$; Table 1), such that rats administered 17β-E_2 entered more peripheral squares than did rats administered vehicle or DPN.

Tamoxifen, compared to vehicle administration, attenuated antianxiety effects in the open field task. Tamoxifen significantly decreased the number of central ($F_{1,72} = 20.95$; $p < 0.01$), but not peripheral ($p = 0.12$), entries compared to vehicle administration.

There was a significant interaction between SERM administration and tamoxifen administration on behavior in the open field task. Rats that were coadministered 17β-E_2, DPN, or coumestrol, but not vehicle, and tamoxifen, had significantly fewer central entries ($F_{3,72} = 2.67$; $p < 0.05$), but no differences in peripheral entries ($p = 0.27$), in the open field compared to rats that were coadministered SERMs and vehicle.

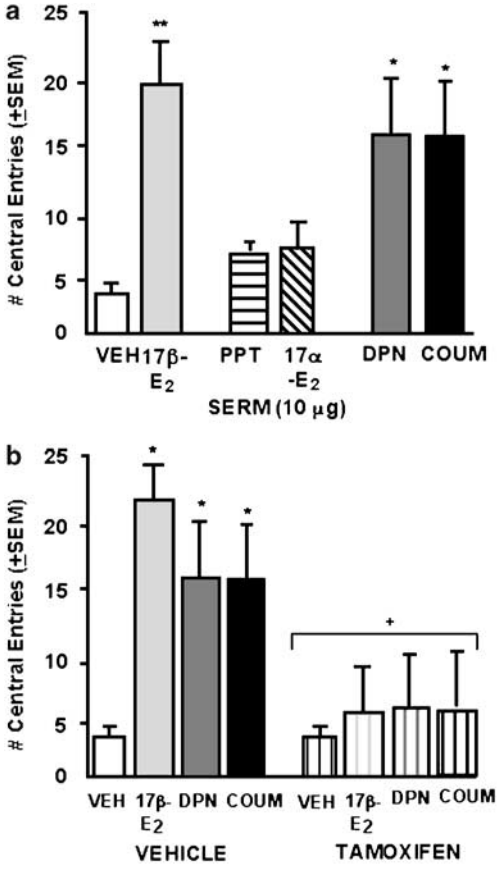

Figure 2 (a) The mean ($\pm$SEM) central entries in the open field of ovx rats administered vehicle, 17β-E_2, PPT, 17α-E_2, DPN, coumestrol (COUM) 48 h before testing ($n = 10$/condition). (b) The mean ($\pm$SEM) central entries in the open field of ovx rats coadministered vehicle (solid bars) or tamoxifen (striped bars) and vehicle, 17β-E_2, COUM, or DPN 48 h before testing ($n = 10$/condition). * above bar indicates a significant difference from vehicle. ** above bar indicates a significant difference from vehicle, PPT, and 17α-E_2. + above grouped bars indicates a significant effect of coadministration of tamoxifen compared to vehicle ($p < 0.05$).

Table 1 Open Field Peripheral Entries, Elevated Plus Maze Open and Closed Arm Entries and Closed Arm Time and Defensive Freezing Task Latencies to Touch Shock Prod and Flinch-Jump Ratings

	Peripheral entries	Open arm entries	Closed arm entries	Closed arm time	Latency to touch shock prod	Flinch-jump ratings
SERM (10 μg)						
Vehicle	73.1±11.5	0.2±0.1	4.4±0.9	298.3±1.2	66.7±13.0	1.8±0.2
17β-E_2	86.8±11.5	2.0±0.4**	5.6±0.8	272.5±3.9**	154.1±50.3	1.6±0.2
PPT	74.8±7.4	0.6±0.2	3.3±0.5	294.4±2.9	115.9±21.9	1.9±0.2
17α-E_2	62.4±9.9	0.5±0.2	3.8±0.4	295.9±1.9	117.5±29.3	1.8±0.2
DPN	105.4±12.1**	1.5±0.3**	5.3±0.6	279.5±3.9**	129.1±55.4	1.6±0.2
Coumestrol	112.9±16.7**	2.3±0.9**	6.2±1.1	274.5±10.8**	61.8±20.2	1.5±0.2
SERM (10 μg) and tamoxifen (10 mg/kg) treatment						
Vehicle+vehicle	65.8±15.1	0.3±0.2	3.1±0.4	297.5±1.7	151.3±42.9	1.6±0.2
Vehicle+17β-E_2	89.9±11.6*	1.8±0.4	5.7±1.1	281.2±3.7*	71.8±24.0	1.9±0.2
Vehicle+coumestrol	95.5±9.5	1.6±0.3	4.3±0.7	274.4±5.2*	92.4±25.1	1.4±0.2
Vehicle+DPN	87.4±11.5	2.0±0.4	6.2±0.7	274.2±5.3*	87.4±25.4	1.7±0.3
Tamoxifen+vehicle	67.8±10.8	0.7±0.3	5.3±0.9	296.0±2.0	97.1±27.9	1.9±0.3
Tamoxifen+17β-E_2	92.6±13.4*	0.4±0.2†	4.5±1.1	298.4±0.7†	38.2±12.1	1.5±0.2
Tamoxifen+coumestrol	76.2±12.0	0.5±0.2†	5.7±1.1	297.4±1.3†	125.9±39.8	1.5±0.2
Tamoxifen+DPN	49.9±11.3	0.4±0.2†	6.7±1.1	298.0±1.1†	68.0±14.2	2.1±0.2

*Significant difference from vehicle ($p<0.05$); **Significant difference from vehicle, PPT, and 17α-E_2 ($p<0.05$); †Significant effect of tamoxifen compared to vehicle ($p<0.05$).

Elevated plus maze. There were antianxiety effects of 17β-E_2 and ERβ-selective SERMs administration in the elevated plus maze. There were differences among groups in the number of entries ($F_{5,54}=4.21$; $p<0.01$; see Table 1) and duration of time spent on the open arms of the maze ($F_{5,54}=5.00$; $p<0.01$; see Figure 3a). Similarly, there was a trend for groups to be different in the number of closed arm entries ($F_{5,54}=2.22$; $p<0.06$) and significant differences among groups for duration spent on the closed arms of the plus maze ($F_{5,54}=5.06$; $p<0.01$; see Table 1). *Post hoc* tests revealed that rats administered 17β-E_2, DPN, or coumestrol made more open arm entries, spent more time on the open arms, and less time on the closed arms of the maze than did rats administered vehicle, PPT, or 17α-E_2. DPN or coumestrol-administered rats also made more entries on the closed arms of the plus maze compared to rats administered PPT or 17α-E_2.

As in Experiment 2, there was a main effect of SERM administration on behavior in the elevated plus maze in Experiment 3. Rats administered 17β-E_2, DPN, or coumestrol spent more time on the open arms ($F_{3,72}=5.21$; $p<0.01$; see Figure 3b), less time on the closed arms ($F_{3,72}=5.28$; $p<0.01$; see Table 1), and tended to make more open arm entries ($F_{3,72}=2.48$; $p<0.06$; Table 1) than did rats administered vehicle. There was no main effect of SERM administration on the number of closed arm entries made ($p=0.11$).

There was a main effect of tamoxifen administration on behavior in the elevated plus maze. Tamoxifen administration significantly decreased the duration spent ($F_{1,72}=54.42$; $p<0.01$), and entries made ($F_{1,72}=21.43$; $p<0.01$), on the open arms and increased the duration spent on the closed arms ($F_{1,72}=50.12$; $p<0.01$) of the maze compared to vehicle. There was no main effect of tamoxifen administration on the number of closed arm entries made ($p=0.27$).

There was a significant interaction between SERM and tamoxifen administration on behavior in the elevated plus maze. 17β-E_2, DPN, or coumestrol, but not vehicle, coadministered with tamoxifen significantly decreased the time spent ($F_{3,72}=7.16$; $p<0.01$) and entries ($F_{3,72}=5.15$; $p<0.01$) on the open arms, and increased the duration spent on the closed arms of the plus maze ($F_{3,72}=7.13$; $p<0.01$). There was no interaction for the number of closed arm entries made ($p=0.30$).

Emergence task. In the emergence task, there were antianxiety effects of 17β-E_2 and ERβ-selective SERMs compared to vehicle or ERα-selective SERMs. There were differences among groups in the latency to emerge from a cylinder ($F_{5,54}=3.28$; $p<0.01$; see Figure 4a). *Post hoc* tests revealed that administration of 17β-E_2, DPN, or coumestrol significantly reduced the latency to emerge from a cylinder compared to vehicle, PPT, or 17α-E_2.

Consistent with results from Experiment 2, there was evidence for antianxiety effects of 17β-E_2, DPN, and coumestrol, compared to vehicle in Experiment 3. There was a tendency for groups to be different for the latency to emerge from a cylinder ($F_{3,72}=2.13$; $p<0.10$; see Figure 4b).

There was a significant main effect of tamoxifen administration on emergence latencies ($F_{1,72}=23.45$; $p<0.01$). Rats administered tamoxifen had significantly longer latencies to emerge from a cylinder than did rats administered vehicle.

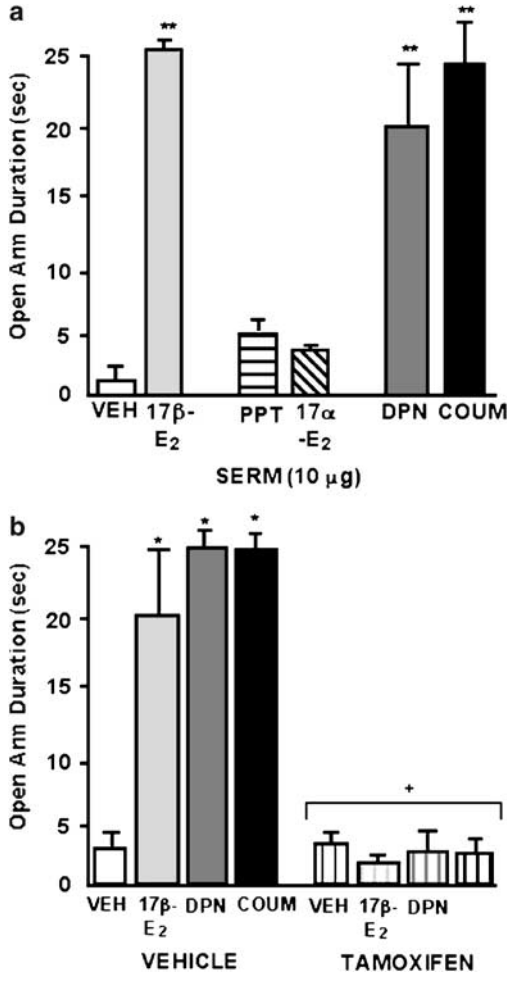

Figure 3 (a) The mean (±SEM) duration spent on the open arms of the plus maze of ovx rats administered vehicle, 17β-E_2, PPT, 17α-E_2, DPN, coumestrol (COUM) 48 h before testing ($n = 10$/condition). (b) The mean (±SEM) duration spent on the open arms of the plus maze of ovx rats coadministered vehicle (solid bars) or tamoxifen (striped bars) and vehicle, 17β-E_2, COUM, or DPN 48 h before testing ($n = 10$/condition). * above bar indicates a significant difference from vehicle. ** above bar indicates a significant difference from vehicle, PPT, and 17α-E_2. + above grouped bars indicates a significant effect of coadministration of tamoxifen compared to vehicle ($p < 0.05$).

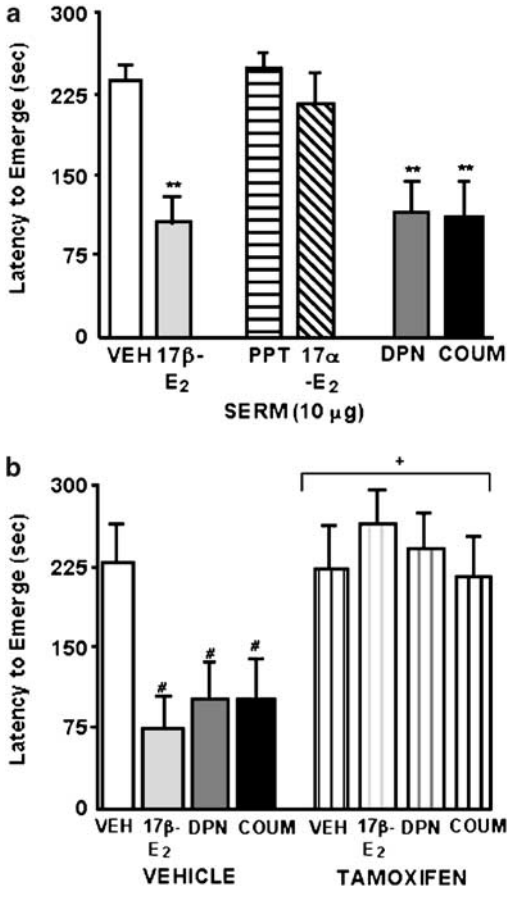

Figure 4 (a) The mean (±SEM) latency to emerge from a dark chamber of ovx rats administered vehicle, 17β-E_2, PPT, 17α-E_2, DPN, coumestrol (COUM) 48 h before testing ($n = 10$/condition). (b) The mean (±SEM) latency to emerge from a dark chamber of ovx rats coadministered vehicle (solid bars) or tamoxifen (striped bars) and vehicle, 17β-E_2, COUM, or DPN 48 h before testing ($n = 10$/condition). * above bar indicates a significant difference from vehicle. ** above bar indicates a significant difference from vehicle, PPT, and 17α-E_2. + above grouped bars indicates a significant effect of coadministration of tamoxifen compared to vehicle ($p < 0.05$). # indicates a tendency to be different compared to vehicle, PPT, and 17α-E_2 ($p < 0.10$).

There was a significant interaction between SERM and tamoxifen administration on emergence latencies. Coadministration of tamoxifen with 17β-E_2, DPN, or coumestrol, but not vehicle, significantly increased the latency to emerge ($F_{3,72} = 2.92$; $p < 0.04$) compared to coadministration of SERMs and vehicle.

Light–dark transition. There were antianxiety effects of 17β-E_2 and ERβ-selective SERMs compared to vehicle or ERα-selective SERMs in the light–dark transition task. There was a tendency for groups to differ in the duration of time spent on the light side of the chamber ($F_{5,54} = 2.02$; $p < 0.01$; see Figure 5a). *Post hoc* tests revealed that administration of 17β-E_2, DPN, or coumestrol increased the time spent on the light side of the chamber compared to vehicle, PPT, or 17α-E_2.

Similar to effects observed in Experiment 2, there was a main effect of SERM administration on behavior in the light–dark transition task in Experiment 3. Rats administered 17β-E_2, DPN, or coumestrol spent more time in the light side of chamber ($F_{3,72} = 5.27$; $p < 0.02$; see Figure 5b) than did rats administered vehicle.

There was a main effect of tamoxifen administration on behavior in the light–dark transition task. Tamoxifen administration significantly decreased the duration spent on the light side of the chamber ($F_{1,72} = 3.97$; $p < 0.05$) compared to vehicle.

Defensive freezing task. In the defensive freezing task, there were antianxiety effects of 17β-E_2 and ERβ-selective SERMs compared to vehicle or ERα-selective SERMs. There were differences among groups in the time spent freezing after a footshock in this task ($F_{5,54} = 17.74$; $p < 0.01$; see Figure 6a).

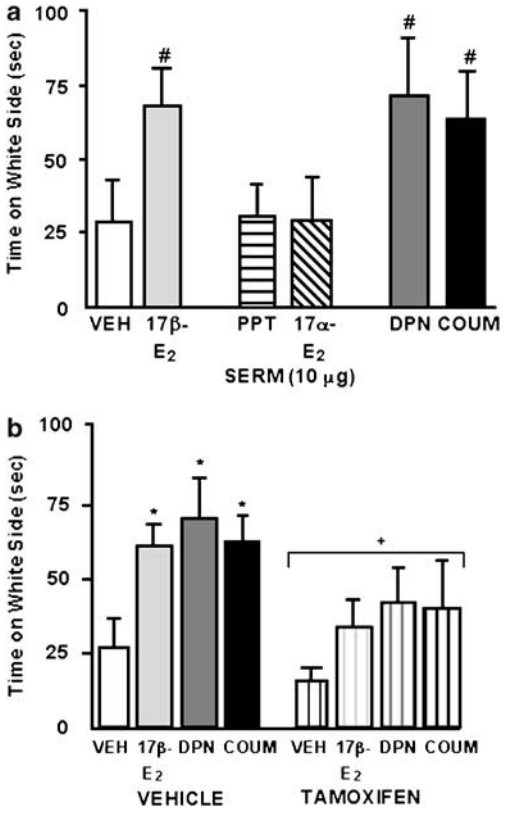

Figure 5 (a) The mean (±SEM) duration spent on the white side of the chamber in the light–dark transition task of ovx rats administered vehicle, 17β-E_2, PPT, 17α-E_2, DPN, coumestrol (COUM) 48 h before testing ($n = 10$/condition). (b) The mean (±SEM) duration spent on the white side of the chamber in the light–dark transition task of ovx rats coadministered vehicle (solid bars) or tamoxifen (striped bars) and vehicle, 17β-E_2, COUM, or DPN 48 h before testing ($n = 10$/condition). * above bar indicates a significant difference from vehicle. ** above bar indicates a significant difference from vehicle, PPT, and 17α-E_2. + above grouped bars indicates a significant effect of coadministration of tamoxifen compared to vehicle ($p < 0.05$). # indicates a tendency to be different compared to vehicle, PPT, and 17α-E_2 ($p < 0.10$).

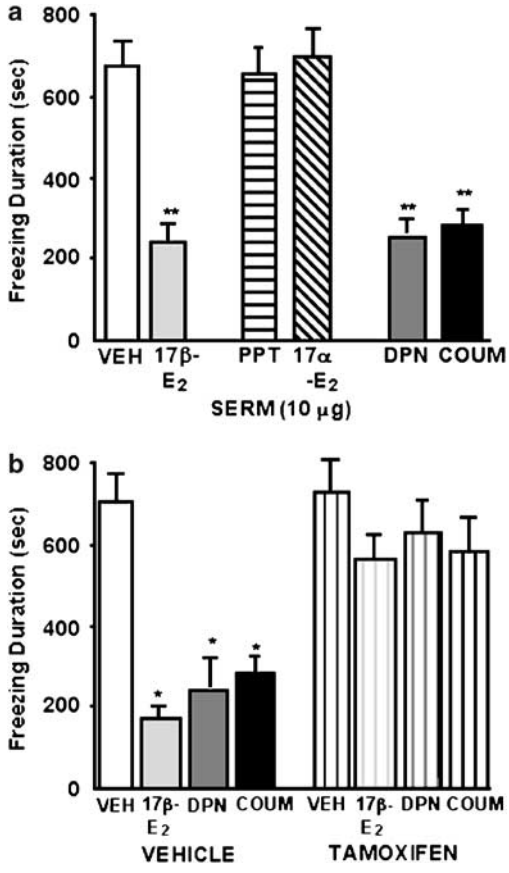

Figure 6 (a) The mean (±SEM) duration spent freezing post-footshock in the defensive freezing task of ovx rats administered vehicle, 17β-E_2, PPT, 17α-E_2, DPN, coumestrol (COUM) 48 h before testing ($n = 10$/condition). (b) The mean (±SEM) duration spent freezing post-footshock in the defensive freezing task of ovx rats coadministered vehicle (solid bars) or tamoxifen (striped bars) and vehicle, 17β-E_2, COUM, or DPN 48 h before testing ($n = 10$/condition). * above bar indicates a significant difference from vehicle. ** above bar indicates a significant difference from vehicle, PPT, and 17α-E_2. + above grouped bars indicates a significant effect of coadministration of tamoxifen compared to vehicle ($p < 0.05$).

Post hoc tests revealed that administration of 17β-E_2, DPN, or coumestrol significantly reduced the time spent freezing compared to vehicle, PPT, or 17α-E_2. Administration of SERMs, except for coumestrol, increased latencies of rats to touch the shock prod compared to vehicle-administration, but significant differences were not observed ($p = 0.40$; see Table 1). Similarly, there were no differences among groups on their flinch-jump reaction to footshock ($p = 0.73$; see Table 1).

As in Experiment 2, there was a main effect of SERM-administration for behavior in the defensive freezing task in Experiment 3 ($F_{3,72} = 18.18$; $p < 0.01$; Figure 6b). Rats administered 17β-E_2, DPN, or coumestrol spent significantly less time freezing after footshock than did vehicle-administered rats.

There was a significant main effect of tamoxifen on freezing behavior in the defensive freezing task. Tamoxifen significantly increased freezing behavior following footshock than did vehicle-administration ($F_{1,72} = 37.21$; $p < 0.01$).

There was a significant interaction between SERM and tamoxifen administration. Coadministration of tamoxifen and 17β-E_2, DPN, or coumestrol, but not vehicle, significantly increased time spent freezing by rats ($F_{3,72} = 4.54$; $p < 0.01$).

In Experiment 3, there was a tendency for SERM treatment to alter touch latencies in the defensive freezing task ($F_{3,72} = 2.42$; $p < 0.07$), such that rats administered vehicle or coumestrol had longer latencies than rats administered 17β-E_2 (see Table 1). There was no significant main effect of tamoxifen treatment ($p = 0.36$), nor an interaction between SERM and tamoxifen treatment ($p = 0.46$), on this measure.

There were no significant main effects of SERM ($p = 0.23$) or tamoxifen administration ($p = 0.52$) or interactions of both treatments ($p = 0.28$) on flinch-jump ratings to footshock in this task.

Vogel punished drinking task. There were antianxiety effects of 17β-E_2 and ERβ-selective SERMs compared to vehicle or ERα-selective SERMs in the Vogel punished drinking task. There were differences among groups in the number of punished (shock-associated) licks made ($F_{5,54} = 6.03$; $p < 0.01$; see Figure 7a). *Post hoc* tests revealed

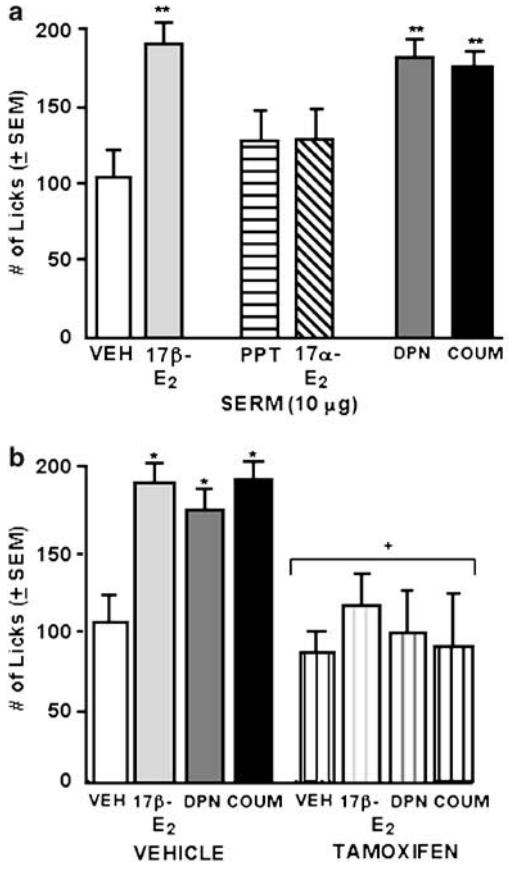

Figure 7 (a) The mean (±SEM) number of punished licks made in the Vogel task of ovx rats administered vehicle, 17β-E_2, PPT, 17α-E_2, DPN, coumestrol (COUM) 48 h before testing ($n = 10$/condition). (b) The mean (±SEM) number of punished licks made in the Vogel task of ovx rats coadministered vehicle (solid bars) or tamoxifen (striped bars) and vehicle, 17β-E_2, COUM, or DPN 48 h before testing ($n = 7$–10/condition). * above bar indicates a significant difference from vehicle. ** above bar indicates a significant difference from vehicle, PPT, and 17α-E_2. + above grouped bars indicates a significant effect of coadministration of tamoxifen compared to vehicle ($p < 0.05$).

that administration of 17β-E_2, DPN, or coumestrol significantly increased the number of punished licks made compared to vehicle, PPT, or 17α-E_2.

Consistent with results from Experiment 2, there was evidence for antianxiety effects of 17β-E_2, DPN, and coumestrol, compared to vehicle in the Vogel punished drinking task ($F_{3,72} = 2.96$; $p < 0.04$; see Figure 7b). *Post hoc* tests revealed that administration of 17β-E_2, DPN, or coumestrol increased punished licks compared to vehicle.

There was a significant main effect of tamoxifen administration on number of punished licks made. Rats administered tamoxifen made significantly less punished licks than did rats administered vehicle ($F_{1,72} = 10.74$; $p < 0.01$).

DISCUSSION

The hypothesis that SERMs with actions at ERβ produce specific antianxiety behavioral effects was supported. 17β-E_2, which binds to both ERα and ERβ, DPN (a highly specific ERβ-selective SERM) and coumestrol (an SERM with greater activity at ERβ than ERα), increased the time spent in the center of the brightly-lit open field and time spent on the open arms of the elevated plus maze compared to vehicle; whereas ERα-selective SERMs, PPT, and 17α-E_2, did not. Similarly, 17β-E_2, DPN, and coumestrol also produced significantly shorter emergence latencies, longer durations spent on the light side in the light–dark transition task, less time freezing in response to shock, and more punished licks than did vehicle, PPT, or 17α-E_2. Additionally, the antianxiety effects of 17β-E_2, DPN, and coumestrol were abrogated by coadministration of the nonselective ER antagonist tamoxifen, but not vehicle. These data suggest that activity at ERβ may be sufficient to produce antianxiety behavior.

The present results support previous findings that suggest that actions at ERβ may have a role in mediating affective behaviors. Studies have shown effects of dietary phytoestrogens, with a greater affinity for ERβ, influence anxiety behavior. Exposure to dietary genistein, an SERM with activity at ERβ, throughout gestation and until postnatal day 75, reduced anxiety behavior of male and female Long-Evans rats in the elevated plus maze (Lephart *et al*, 2002; Lund and Lephart, 2001a). In contrast, 18 days of exposure to dietary phytoestrogens increased anxiety behavior and stress hormone levels of male rats. Concentrations of genistein or daidzein, which have greater activity at ERβ than ERα, reduced time spent in social interaction with a conspecific and open arm activity in the plus maze, and significantly elevated stress-induced corticosterone concentrations (Forsling *et al*, 2003; Hartley *et al*, 2003). The differences in these findings may reflect the duration and/or concentration of exposure to dietary phytoestrogens and the resulting effects at ERβ and/or ERα. Indeed, E_2 dosage, duration of exposure, and exposure to stress, are factors that influence whether E_2 has antianxiety and/or anxiogenic effects (Walf and Frye, 2005). Using a paradigm analogous to the present experiments, we have shown that 17β-E_2 and ERβ-selective SERMS reduce depressive behavior (immobility in the forced swim test) compared to ERα-selective SERMs or vehicle (Walf *et al*, 2004). As further support of the role of ERβ in affective behavior, ERβ knockout mice have increased anxiety behavior in the elevated plus maze compared to that observed in wild-type mice (Imwalle *et al* 2005; Krezel *et al*, 2001) and ERα knockout mice (Krezel *et al*, 2001). Thus, the extent to which SERMs activate ERβ, more than ERα, may influence the nature of their effects on affective behavior.

In contrast, ERα may have a more prominent role in reproduction. Although SERMs with greater activity at ERα, PPT, and 17α-E_2, did not alter anxiety behavior, they did facilitate lordosis behavior more than vehicle, and in a manner comparable to 17β-E_2. These data indicate that PPT and 17α-E_2 were available to the brain and could produce specific behavioral effects. ERα has been localized to the hypothalamus, an important brain region for sexual receptivity. E_2-facilitated receptivity of rats is blocked by antisense oligonucleotides for ERα (not ERβ), and does not occur in ERβ (but does occur in ERα) knockout mice (Apostolakis *et al*, 2000; Ogawa *et al*, 1996, 1998, 1999). Further, ERα knockout mice are anovulatory, have dis-

rupted luteinizing hormone secretion, and do not respond to trophic actions of E_2 on uterine tissues. Although ERα knockout mice have reduced ovulatory capacity, they are fertile (Hewitt and Korach, 2003). Our data confirm that actions at ERα are important for lordosis.

E_2 also has well known effects on activity and/or arousal of people and animals (Smith, 1994). Proestrous rats or mice, or ovx rats administered E_2, demonstrate more spontaneous motor activity (Becker *et al*, 1987; Joyce and Van Hartesveldt, 1984; Morgan and Pfaff, 2002), which may disrupt performance in some behavioral tasks. As well, E_2, particularly in the higher range of concentrations (25 μg, s.c. to mice), enhances arousal (Morgan and Pfaff, 2002), which may influence performance. In the present experiments, there was some evidence for ERβ-selective SERMs that had antianxiety effects to also enhance general activity measures in the same tasks, which need to be considered as a possible confound in interpretation of the antianxiety behavioral effects of these compounds. In the open field, 17β-E_2, DPN, and coumestrol increased the number of central and peripheral entries. In the elevated plus maze, 17β-E_2, DPN, and coumestrol, increased time spent on the open arms, decreased time spent on the closed arms, and increased the number of entries to both. In contrast, there were no effects of SERMs on other measures of activity or arousal. SERMs did not alter the number of beam breaks during a 5-min spontaneous activity task, latency to touch the shock-associated prod, or influence flinch/jump responses to shock. Together, these data of SERMs' effects to alter some measures of motor behavior, but not alter latency to touch the shock-associated prod and flinch/jump responses to shock, suggest that the antianxiety effects observed in the present study are influenced, but not solely due to changes in motor activity and/or arousal. Previous research suggests that E_2's actions at ERα may be essential for E_2-enhanced activity of mice. For example, running wheel activity in E_2-primed mice lacking ERα is attenuated compared to their wild-type controls; however, there are no differences between ERβ knockout and their wild-type controls for running wheel activity (Ogawa *et al*, 2003; Pfaff *et al*, 2002). These data suggest that further investigation of SERMs' effects on activity and arousal measures are needed to clarify their role in these and other functional effects.

Although the present findings that ERβ-active SERMs have antianxiety effects are intriguing, the limitations of the findings should be considered. First, the brain areas that mediate E_2's effects on anxiety have not been established, although the hippocampus, amygdala, and/or septum have been implicated (Frye and Walf, 2002, 2004, 2005; Molina-Hernandez and Tellez-Alcantara, 2001). As such, peripheral dosing with SERMs was utilized to characterize the effects of these compounds in the battery of affective tasks utilized in this study. Second, the concentration-dependent and/or time course effects of SERMs were not addressed in the present study. All rats were administered 10 μg SERMs 48 h before testing and plasma or central concentrations of these compounds were not determined. This limitation precludes the conclusion that there is no effect of ERα-selective SERMs for affective behavior because the regimen employed may have produced insufficient concentrations of ERα-selective agonists at the time of testing. For example, given the greater distribution of ERβ compared to ERα in the hippocampus (Shughrue *et al*, 1997), a higher concentration of ERα-selective SERMs may be necessary for them to influence anxiety behavior. However, the ERα-selective SERM regimen utilized did facilitate lordosis and other reports have demonstrated behavioral effects of lower and higher dosages of both ERα and ERβ-selective SERMs (Luine *et al*, 2003; Overstreet *et al*, 2004). Perhaps, the ERα-selective SERMs utilized in the present study were effective in the hypothalamus, but not limbic regions, to modulate behavioral effects because of the higher density of ERα in the hypothalamus. Additionally, the potential modulatory role of ERβ on ERα is yet another factor that precludes the conclusion that ERα is not an integral substrate for affective behavior (Lindberg *et al*, 2003). Third, the specific mechanisms of action for E_2 were not clearly identified in these studies. E_2 has been shown to alter hypothalamic–pituitary–adrenal axis activity, and this may underlie some of the differences observed in affective tasks (Walf and Frye, 2005). It may be that some of the differences, or lack thereof, in the tasks utilized in the present study (ie no differences in latencies to the shock prod or flinch/jump responses and no interactive effects of tamoxifen and SERMs in the light–dark and Vogel tasks) were due to variations in the stress responses of rats. Other laboratories have reported that rats administered DPN have reduced plasma corticosterone levels 30 min following elevated plus maze testing compared to rats administered 17β-E_2 and PPT, but not vehicle, administration (Lund *et al*, 2005). Future experiments could investigate whether E_2's actions via ERs for its effects on anxiety behavior are modulated by activity of the hypothalamic–pituitary–adrenal axis. In support, coadministration of E_2 with an ER antagonist, tamoxifen, attenuates effects of E_2 to reduce the adrenocorticotropin hormone and corticosterone response to restraint stress (Young *et al*, 2001). In the present study, the antianxiety behavior produced by ERβ-active SERMs was blocked by administration of the nonspecific, but effective, ER antagonist, tamoxifen. Tamoxifen is not a pure ER antagonist and, in some dosages, tamoxifen may exhibit agonist properties, which may be due to actions at ERα, whereas its antagonistic properties may be due to actions at ERβ (Watanabe *et al*, 1997). There was no evidence for nonspecific effects of tamoxifen in the present study. Although the use of a pure ER antagonist, such as ICI 182,780 might be informative, it does not readily cross the blood–brain barrier and must be administered centrally. The purpose of this study was to determine whether effects of peripherally administered SERMs can be attenuated by ER-blockade across all brain regions. In addition, ICI 182,780 may be inactive in the hippocampus (Gu *et al*, 1999), as such, this precluded the use of this antagonist. In future experiments, antisense oligonucleotides for ERα and ERβ, which block the transcriptional process, and appropriate controls, can be administered directly to putative brain areas. Behavioral effects concomitant with verification of ER blockade will help establish which particular brain regions are critical for ERβ-mediated antianxiety effects.

The findings that ERβ-active SERMs produce antianxiety effects have particularly intriguing implications. More women than men suffer from anxiety-related disorders

(Pigott, 1999; Seeman, 1997; Wittchen and Hoyer, 2001). Some pharmacotherapies for anxiety are addictive and others, such as selective serotonin re-uptake inhibitors, have long-latencies to act and can produce sexual side effects (Lane, 1997). There is evidence that HT with E_2 may have antianxiety effects for some, but not all, women (Arpels, 1996; Campbell and Whitehead, 1977; Pearlstein *et al*, 1997; Pigott, 1999; Smith *et al*, 1995; Schmidt *et al*, 1998; Torizuka *et al*, 2000). However, a substantial criticism about HTs with E_2 are their potential proliferative effects on breast and/or uterine tissues, which are primarily mediated via ERα (Gustafsson, 2003; Hillisch *et al*, 2004). Recent reports indicate that administration of ERβ-selective SERMs, in higher concentrations than were used in the present study, do not demonstrate proliferative effects in uterine tissue of ovx rats (McBride *et al*, 2004). Together, these data suggest that it may be feasible to dissociate the beneficial antianxiety effects of SERMs from their negative proliferative effects on reproductive organs. Notably, there is evidence that some beneficial effects of SERMs on cognitive performance may require actions at ERα and/or ERβ (Lund and Lephart, 2001b; Luine *et al*, 2003; Rhodes and Frye, 2005); however, this remains to be established.

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