Anxiolytic Effects and Neuroanatomical Targets of Estrogen Receptor-β (ERβ) Activation by a Selective ERβ Agonist in Female Mice

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The dichotomous anxiogenic and anxiolytic properties of estrogens have been reported to be mediated by two distinct neural estrogen receptors (ER), ERα and ERβ, respectively. Using a combination of pharmacological and genetic approaches, we confirmed that the anxiolytic actions of estradiol are mediated by ERβ and extended and these observations to demonstrate the neuroanatomical targets involved in ERβ activation in these behavioral responses. We examined the effects of the biologically active S-enantiomer of diarylpropionitrile (S-DPN) on anxiety-related behavioral measures, the corresponding stress hormonal response to hypothalamo-pituitary-adrenal axis reactivity, and potential sites of neuronal activation in mutant female mice carrying a null mutation for ERβ gene (βERKO). S-DPN administration significantly reduced anxiety-like behaviors in the open field, light-dark exploration, and the elevated plus maze (EPM) in ovariectomized wild-type (WT) mice, but not in their βERKO littermates. Stress-induced corticosterone (CORT) and ACTH were also attenuated by S-DPN in the WT mice but not in the βERKO mice. Using c-fos induction after elevated plus maze, as a marker of stress-induced neuronal activation, we identified the anterodorsal medial amygdala and bed nucleus of the stria terminalis as the neuronal targets of S-DPN action. Both areas showed elevated c-fos mRNA expression with S-DPN treatment in the WT but not βERKO females. These studies provide compelling evidence for anxiolytic effects mediated by ERβ, and its neuroanatomical targets, that send or receive projections to/from the paraventricular nucleus, providing potential indirect mode of action for the control of hypothalamo-pituitary-adrenal axis function and behaviors. (***Endocrinology*** **153: 837–846, 2012)**

Estrogens play an important role in the central nervous system and modulate several functions including mood, fear, anxiety, depression, cognition, and memory in humans and laboratory animals (1–3). Depending on the stage of the reproductive cycle, estrogens have been reported to exhibit anxiogenic or anxiolytic properties in rodents. Elevated levels of estradiol during proestrus or exogenous administration of estradiol to ovariectomized females elicit anxiolytic actions (4–6). Removal of endogenous estrogens by ovariectomy enhances anxiety-like behaviors, whereas estradiol replacement has been reported to increase or decrease behavioral measures of anxiety in rodents (7).

This dichotomous neurobiological response to estrogens has been attributed to their actions on two distinct estrogen receptor (ER) systems, ERα and ERβ. In the brain, ERα plays a critical role in regulating reproductive neuroendocrine function, whereas ERβ is thought to be important in regulating nonreproductive functions (8–11). Support for ERβ's role in anxiety, stems from the studies on anxiety-related behaviors in female rats, treated with an ER subtype-selective agonist (12). Pharmacolog-

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Abbreviations: BST, Bed nucleus of the stria terminalis; CORT, corticosterone; DPN, diarylpropionitrile; EPM, elevated plus maze; ER, estrogen receptor; βERKO, null mutation for ERβ gene; HPA, hypothalamo-pituitary-adrenal; MEAad, anterodorsal medial amygdala; OT, oxytocin; OTR, open to total ratio; PVN, paraventricular nucleus; R-DPN, R-enantiomer of DPN; S-DPN, S-enantiomer of DPN; WT, wild type.

ical administration of the ERβ-specific agonist, diarylpropionitrile (DPN), to ovariectomized female rats decreased anxiety-related behaviors measured in the open-field arena, elevated plus maze (EPM) and light-dark box. The ERα selective agonist, propylpyrazoletriol, however, was found to be anxiogenic in the same behavioral measures (12). In another study, DPN-treated ovariectomized rats exhibited decreased anxiety in the open field and less depressive-like behavior in the forced swim test (13, 14). Furthermore, gonad-intact, female ERβ knockout mice (βERKO) show increased anxiety compared with their wild-type (WT) littermates, whereas ERα knockouts do not differ from their WT littermates in these parameters (15, 16). Administration of DPN to ovariectomized females increased open-field entries and the time spent in open arms in the EPM in WT mice but not the βERKO mice, indicating a role for ERβ in anxiety responses (17). The anxiolytic behavioral effects of ERβ selective agonist DPN (18) and WAY-200070 (Wyeth, Princeton, NJ; Ref. 19) have also been reported in βERKO mice.

Recent reports indicate that the widely used ERβ-subtype specific ligand, DPN, is a racemic mixture of two enantiomers, S-DPN and R-DPN (20). Compared to R-DPN, S-DPN has a higher relative binding affinity to ERβ (6.7-fold) and is a potent activator of transcription *in vitro* (21). Furthermore, S-DPN treatment attenuated the stress-induced reactivity of the hypothalamic pituitary adrenal (HPA) axis, as demonstrated by a decrease in plasma corticosterone (CORT) and ACTH levels and immediate early gene activation of c-fos in the paraventricular nucleus (PVN) of the hypothalamus, one of the regions containing high levels of ERβ expression (22). In addition to the high expression levels in the PVN, ERβ is also expressed in other brain regions such as the olfactory bulb, cerebral cortex, septum, preoptic area, bed nucleus of the stria terminalis, amygdala, thalamus, ventral tegmental area, substantia nigra, dorsal raphe, locus coeruleus, hippocampus, and cerebellum (23). The interactions between these brain regions and the mechanism(s) by which the ERβ-containing neurons in these regions influence HPA axis reactivity to anxiety remain(s) unclear. To address this question, we used S-DPN as a pharmacological tool in conjunction with βERKO mice to examine not only ERβ action on anxiety-related behavioral measures and the corresponding stress hormonal response to HPA axis reactivity, but also the neuroanatomical targets of ERβ action using c-fos mRNA expression as a marker of stress-induced neuronal activation.

Materials and Methods

Animals

Heterozygous ERβ female and male mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and a breeding colony was established at Baylor College of Medicine. The mice used in the current studies were isogenic on C57BL/6J background (>10 generations backcrossed) and were obtained from breeding heterozygous female and male mice. The offspring of this breeding were genotyped by PCR analyses of tail DNA, using primers as described (24). βERKO mice and their WT littermates were housed (two to five per cage) in a room with a 12-h light, 12-h dark cycle (lights on at 0600 h, off at 1800 h) with *ad libitum* access to a soy-free diet (modified AIN-93G, DYET no. 101591; Dyets, Inc., Allentown, PA) and water. Behavioral testing was performed on female βERKO mice and their WT littermates between 0900 h and 1500 h. All procedures were approved by the Baylor College of Medicine Animal Care and Use Committee and followed the National Institutes of Health Guidelines.

Neurological screen

The neurological screen (25) used to identify the obvious behavioral phenotype of the WT and βERKO littermates was adapted from the Irwin Screen (26). Each mouse was placed in an empty cage and observed for 3 min. Several spontaneous behavioral characteristics (*i.e.* wild running, freezing, licking, jumping, and sniffing) were examined. Limb splaying reflexes exhibited by the mouse in response to rapid vertical and horizontal cage movement, the righting reflex when turned on their backs, ear twitch, whisker touch, and eye blink responses upon touching with a cotton swab were assessed. Simple motor responses were examined using the vertical pole test. Each mouse was placed on a cloth tape-covered metal rod held horizontally. The rod was then slowly moved to a 45° position and finally to an upright vertical position. The time a mouse stayed on the rod was recorded for a maximum of 60 sec. Other physical characteristics such as the presence of whiskers, bald hair patches, palpebral closure, exophthalmos, and piloerection were observed and recorded.

Hormone treatments

Mice at 3–4 months of age were ovariectomized under isoflurane anesthesia. Two weeks after ovariectomies, the animals were handled daily (5 min per animal) by the same experimenter. The animals were administered a single sc injection of hydroxypropyl β-cyclodextran [vehicle; 27% (wt/vol) in saline; CTD Inc., High Springs, FL] or the S-DPN (1.0 mg/kg in vehicle) daily for 7 d. Purified S-DPN was obtained from the racemic DPN as previously described (21). All daily injections were administered around 0800 h, and the animals underwent behavioral testing on d 4–7, 4 h after the treatment. Each mouse underwent testing in one behavioral paradigm per day in the order described below. To minimize the possibility of altered behaviors due to previous testing experience and to facilitate blood and tissue collection following anxiety-like behavioral tests, our behavioral tests ranged from least invasive to the most invasive, after the behavioral test battery of McIlwain *et al.* (25, 27). A constant white noise (55 dB) and light intensity (800 lux) were maintained in the room while testing and during acclimatization for 60 min before testing.

Locomotor activity in the open field

Animal activity in the open field was analyzed on d 4 of treatment. Mice were placed individually in the center of a Plexiglas open field arena (40 × 40 × 30 cm) and were allowed to explore for 30 min, and the activity in the open field was quantitated by

a computer-operated Digiscan optical animal activity system (RXYZM; Omnitech Electronics, Dartmouth, Nova Scotia, Canada) containing 16 photoreceptor beams on each side of the arena, which divides the arena into 256 equally sized squares. The total distance (locomotor activity), the vertical activity (interruption of upper beans by rearing posture), and the center time and center distance (*i.e.* time spent and the distance traveled in the center of the arena) were recorded.

Light-dark exploration

This exploration test consisted of a polypropylene chamber (44 × 21 × 21 cm) unequally divided into a larger, brightly illuminated open compartment (clear polypropylene) and a smaller, dark compartment (in dark polypropylene), connected by a small opening. Mice were placed in the illuminated chamber and allowed to move freely between the two chambers for 10 min. The latency to enter the light and dark chambers, the time spent in the chambers, and the total number of transitions were measured using the Psion Workabout (Psion Teklogix, Hebron, KY) and analyzed. Transfer of all four paws of an experimental animal from one chamber to the other was considered as one transition event.

Marble-burying behavior

This test used to evaluate active coping and stress-provoked, anxiety-like behavior (28, 29). A clean cage (27 × 16.5 × 12.5 cm) with sanichip bedding (depth 4.5 cm) containing 20 black glass marbles (15 mm diameter) evenly spaced on the top of the bedding in a 5 × 4 arrangement was used. A 5-cm space on one lateral side of the cage was left marble free. Mice were gently placed individually in the marble-free area and left undisturbed for 30 min. At the end of the experiment, the mice were removed and the number of buried marbles was counted. A marble was considered buried when it was covered at least two thirds by the bedding.

Elevated plus maze

The EPM was constructed of plexiglas with two open arms (30 × 5 cm) and two enclosed black arms (30 × 5 × 15 cm) at an elevation of 50 cm above the floor. The arms of the maze form a cross with the two open arms facing each other. The maze was cleaned with 70% ethanol solution after each session and allowed to dry between the sessions. Anxiety-like behavior was measured by placing the mice in the center of the junction of the arms of the maze facing an open arm and the behavior analyzed for 10 min. The numbers of entries into the open and closed arms and the time spent exploring the open and closed arms were recorded and analyzed using the ANY-maze software (Stoelting Co., Wood Dale, IL). The changes in anxiety-like behavior were calculated by dividing the number of entries into the open arms by the total number of entries into all four arms [open to total ratio (OTR) for entries] or by dividing the amount of time spent in the open arms by the amount of time spent in all four arms (OTR for time). The time spent in the center platform not exploring any of the arms was not included in these calculations.

Plasma hormone measurement

Mice were euthanized via decapitation after the EPM test (~15 min from the start of EPM) and trunk blood collected into ice-chilled tubes containing 0.5M EDTA and 4 μg/ml aprotinin (Sigma Aldrich Chemicals, St. Louis, MO). Blood was centrifuged at 4 C, and plasma was removed and stored at −70 C until assayed for CORT and ACTH.

CORT was measured using a RIA (30). Briefly, plasma was diluted in 0.01 M PBS (1:25) and corticosteroid binding globulin inactivated by incubation at 65 C for 1 h. After overnight incubation of the samples (20 μl) and standards (5–700 ng/ml), with antiserum (rabbit anti-CORT: MP Biomedical, Solon, OH) and [^{3}H] CORT (PerkinElmer, Boston, MA) in 0.1% gelatin in 0.01 M PBS at 4 C, dextran-coated charcoal (1.0 ml) was used to separate free CORT from antibody bound CORT. After centrifugation, the supernatant containing antibody-bound CORT was mixed with 4 ml of scintillation fluid and counted in a Packard 2900 TR liquid scintillation counter (Packard Bioscience Co., Meriden, CT). The intraassay and interassay variance as measured by internal quality controls was 4.5 and 7.8% respectively.

For the ACTH assay, plasma was diluted in PBS-albumin (0.01 PBS, 0.09% NaCl, 0.1% albumin, 100,000 kIU aprotinin per liter). The samples and standards (5–2000 pg/tube) were incubated overnight at 4 C with antiserum (rabbit anti-ACTH; Immunostar, Hudson, WI) and 2% normal rabbit serum. The tracer, [^{125}I]ACTH (1–39) (Amersham, Piscataway, NY) in PBS-albumin (100 μl), was added and incubated overnight at 4 C. On the following day, goat antirabbit γ-globulin (Calbiochem, La Jolla, CA) was added to all samples (excluding totals) and incubated overnight at 4 C. On d 4, 3 ml of PBS-albumin was added to the samples and then centrifuged (>1000 × *g*). The pellets were counted with a Packard Cobra II γ-counter (Packard Bioscience). The intraassay and interassay variance as measured by internal quality controls was 3.8 and 6.1%, respectively.

In situ hybridization

In situ hybridization for c-fos was performed on 16-μm-thick, coronal brain slices of animals (21, 31). Briefly, tissue sections were thawed at room temperature, fixed with 4% formaldehyde, acetylated with 0.25% acetic anhydride, delipidated in chloroform, dehydrated in graded alcohols, and air dried. A 48-base oligonucleotide probe (5′-gca gcg gga gga tga cgc ctc gta gtc cgc gtt gaa acc cga gaa cat-3′) complementary to the coding region of c-fos mRNA was 3′ end labeled with [^{35}S]dATP (~1 × 10^7 cpm/pmol) using terminal deoxynucleotide transferase (New England Biolabs, Beverly, MA). Sections were hybridized with labeled probe (1.5 × 10^7 cpm/ml) in hybridization buffer (0.6 M NaCl, 10 mM Tris, 1× Denhart's, 1 mM EDTA, 10% dextran sulfate, 50% formamide, 0.1 mg/ml salmon sperm DNA, 0.5 mg/ml total yeast RNA, 0.05 mg/ml yeast tRNA, and 0.1% sodium dodecyl sulfate) for 16 h at 40 C in a humidified incubator. Sections were washed in 2× standard saline citrate to remove unbound probe and nonspecific hybridization removed by a series of washes of increasing stringency (final stringency at 2× standard saline citrate, 45 C, 50% formamide). For autoradiographic detection of hybridization, slides were exposed to autoradiographic film for 10 d, slides dipped in Kodak NTB3 emulsion (Rochester, NY), dried, exposed in the dark for 14 d, and emulsion developed in Dektol (Kodak). The sections were counterstained with cresyl violent, dehydrated, and coverslipped with DPX mountant (EM Sciences, Fort Washington, PA).

Autoradiograms were analyzed for density using a video camera (Sony XC-77; Tokyo, Japan) attached to a Nikon lens (Melville, NY). Scion Image (Frederick, MD) was used to count density per a fixed region encompassing the selected area. For each

section, background density was obtained from a region adjacent and subtracted from the measurement. Bilateral measurements were obtained from four separate sections and averaged to obtain the value for each individual animal.

The density of hybridization signal was determined from emulsion-coated slides using a video camera (Sony XC-77) connected to a Zeiss axioplan microscope (New York, NY). Images were imported with a QuickCapture framegrabber board (Data Translation Inc., Marlboro, MA) and a Dell computer using Scion Image software. Sections were atlas matched (32), and each individual brain region was identified by examining cresyl violet stained tissue under bright-field microscopy. Silver grains were then visualized by dark-field microscopy with a ×20 objective. Under dark field, microscope images encompassing the following defined brain regions were captured: cingulate cortex, lateral septum, bed nucleus of the stria terminalis (BST), medial preoptic area, PVN, hippocampus, central amygdala, anterodorsal medial amygdala (MEAad), dorsomedial nucleus of the hypothalamus, and arcuate nucleus. Images were inverted using Scion Image software and hybridization density was determined per fixed area. Microscopic light intensity was held constant in all analyses. Two measurements were taken bilaterally from three to five different sections for each rat (depending on brain region) by an investigator blind to treatments using the following criteria: grain density must be greater than 5 times background (background determined by counting grains over a cellular area); and a visible nucleus must have been identified by cresyl violet staining. The means of the sections were averaged together to give the mean value for each individual brain region for each individual animal.

Statistical analysis

Results are presented as means (±SEM). Data were analyzed using StatView statistical analysis software (Abacus Concepts, Inc., Berkeley, CA) or Prism (GraphPad Software Inc., San Diego, CA). For each significant one-way ANOVA, *post hoc* comparisons were made using Dunn's method for the comparison of all groups *vs*. the control group or Tukey/Kramer test for multiple comparisons. For studies examining c-fos mRNA, each brain area was examined individually with treatment. In all cases, $P < 0.05$ was considered statistically significant.

Results

βERKO mice do not exhibit gross phenotypic abnormalities

Individual assessment of βERKO mice and their WT littermates demonstrated no obvious abnormal physical

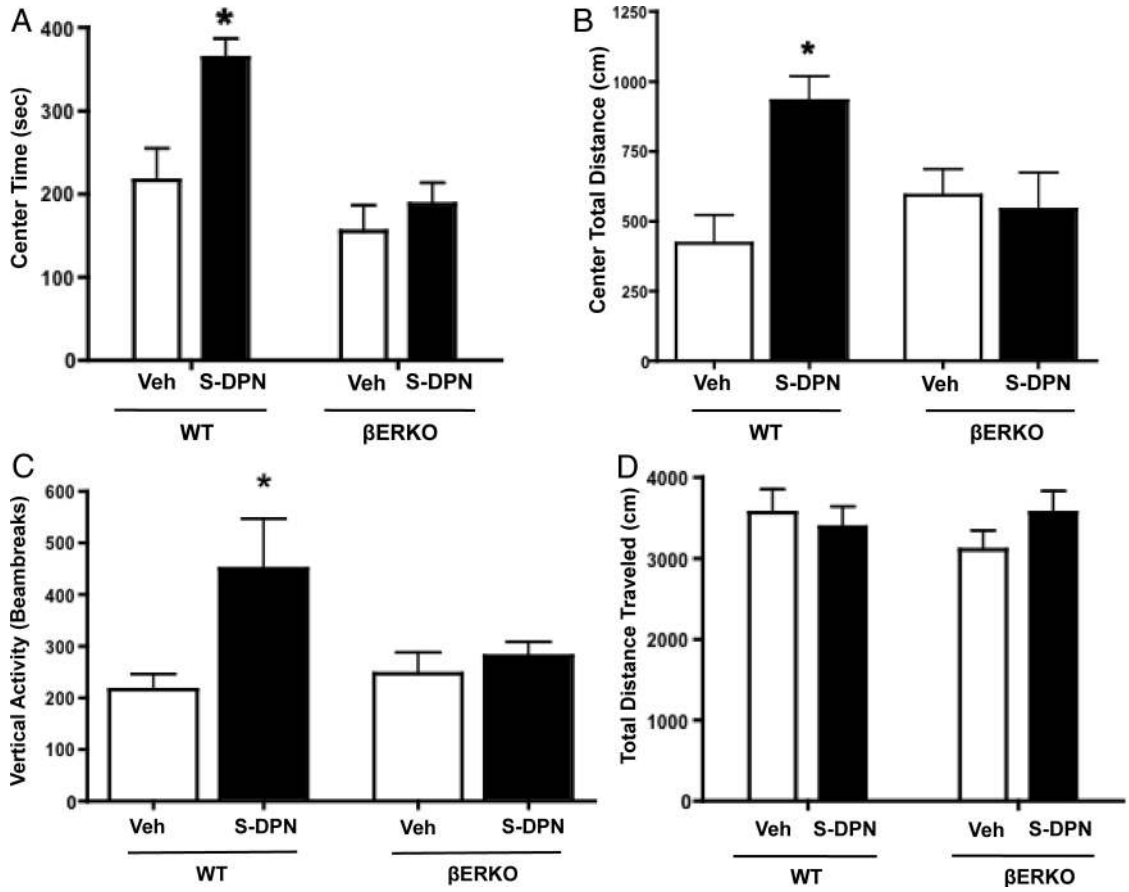

FIG. 1. The open-field activity of female WT and βERKO mice. Ovariectomized animals received daily injections of S-DPN or vehicle (Veh) for 4 d and before behavioral evaluation in the open field. The anxiety-like behavior displayed by both the genotypes, as seen in the time spent (A) and the distance traveled (B) in the center of the open field, was significantly reduced by S-DPN treatment in the WT but not the βERKO mice. C, WT mice that received S-DPN treatment also demonstrated an increase in exploratory behavior as evidenced by an increase in vertical rearing activity. No effects of treatment on both genotypes were observed in the locomotor activity as assessed by total distance traveled (D). *, Significant difference ($P < 0.05$) compared with the vehicle treatment in WT mice. Data are expressed as mean ± SEM, n = 9–12 per group.

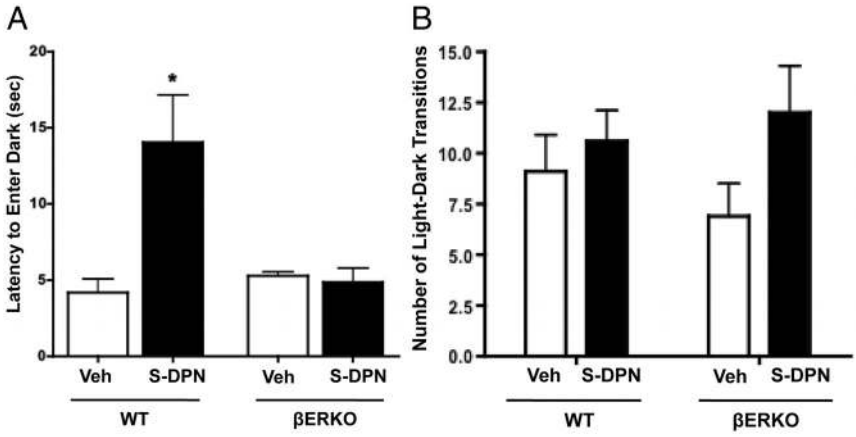

FIG. 2. Exploratory activity in light-dark test for anxiety-related responses. Ovariectomized WT and βERKO mice received daily injections of S-DPN or vehicle (Veh) for 5 d before examination in the light-dark exploratory task. A, The total number of transitions between chambers remained unaffected by S-DPN in both genotypes. B, S-DPN treatment caused a decrease in the anxiety-related response in WT but not the βERKO mice, as measured by the time to enter the dark chamber. *, Significant difference ($P < 0.05$) compared with the vehicle treatment in WT mice. Data are expressed as mean ± SEM for total number of transitions (A) and for latency to enter the dark side (B), n = 9–12 per group.

characteristics such as poorly groomed fur, bald patches in the coat, exophthalmos, palpebral closure, piloerection, or absence of whiskers. The gross neurological functions also appeared to be unimpaired because the animals exhibited no abnormal spontaneous behaviors like wild running, constant circling, jumping, freezing, excessive grooming, sniffing, or hunched body posture while walking. βERKO mice exhibited normal postural reflex by extending all four legs and maintaining an upright and balanced position in response to the rapid cage movement. No significant difference between the genotypes was evident in the rapid righting reflex. The eye blink, ear twitch, or whisker-touch reflexes were also indistinguishable between the genotypes. Motor responses on the vertical pole test were unaffected in both the genotypes.

ERβ receptor mediates anxiety-related behaviors in the WT mice but not the βERKO mice

A role for ERβ in anxiolytic responses of estradiol in rats and mice has been previously demonstrated (11, 12, 18, 21). As a prelude to the determination of the neural site(s) of ERβ modulation of anxiety behaviors, we examined S-DPN effects on anxiety-related behaviors in βERKO mice and their WT littermates in three behavioral paradigms: open field activity, light/dark exploration, and EPM test.

Open-field activity

In the WT mice, S-DPN treatment significantly increased the time spent in the center of the open arena (Fig. 1A; $P < 0.05$), exploratory activity in the center of the arena (Fig. 1B; $P < 0.05$), and vertical rearing activity (Fig. 1C; $P < 0.05$), compared with the vehicle treatment. S-DPN treatment, however, had no significant effect on similar behaviors compared with the vehicle treatment in βERKO mice (Fig. 1, A–C). However, in the assessment of overall locomotor activity, S-DPN-treated βERKO female mice engaged in similar overall activity, as evidenced by the total distance traveled, compared with the vehicle control and S-DPN-treated WT mice (Fig. 1D).

Light-dark exploration

βERKO mice and their WT littermates were examined for their anxiety-related responses in the light-dark box using two measures, *i.e.* the latency to enter the dark chamber and the total number of transitions between the light and dark chambers (Fig. 2). S-DPN treatment significantly increased the latency of the WT mice to enter the dark chamber in the light-dark box, compared with the vehicle controls (Fig. 2A; $P < 0.05$). However, the latency to enter the dark chamber did not significantly differ between the treatment groups in βERKO mice (Fig. 2A). No significant difference in the total number of transitions between the light and dark chambers, without or with S-DPN treatment, was evident in both WT and βERKO littermates (Fig. 2B), suggesting that the tendency of mice to explore novel environment remained unaffected.

Marble-burying behavior

To evaluate whether the presence of novel objects, *i.e.* glass marbles, can induce a behavioral burying response in βERKO mice toward the objects, we examined their marble-burying behavior and compared it with that of the WT littermates. No significant differences were observed in marble-burying behaviors between the genotypes treated with vehicle (Fig. 3), suggesting that the novel presence of the glass marbles did not induce anxiety-related behaviors in either genotype. In addition, S-DPN treatment had no significant effect on the marble-burying behaviors on either of the genotypes (Fig. 3).

Elevated plus maze

When tested on the EPM, WT mice treated with S-DPN demonstrated an increase in open arm exploration, compared with vehicle-treated controls. This was evidenced by the significantly higher OTR values for the time spent in (Fig. 4A; $P < 0.05$) and entries into the open arm (Fig. 4B;

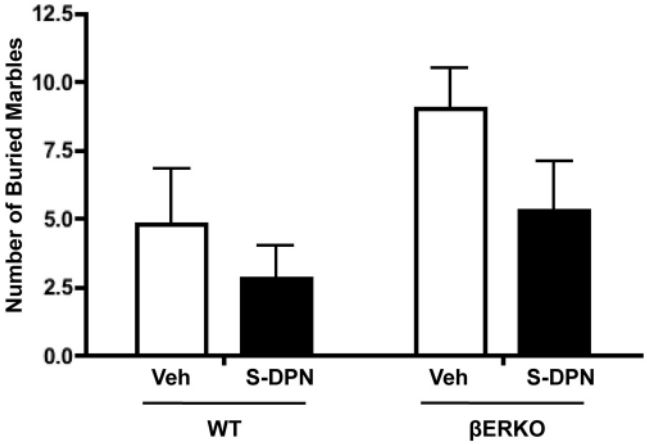

FIG. 3. Marble-burying behaviors in WT and βERKO mice. Both genotypes received daily injections of S-DPN or vehicle (Veh) for 6 d before behavioral assessment in the marble-burying task. No significant differences were observed between the genotypes and the treatments.

$P < 0.05$) by the S-DPN-treated WT mice, compared to the vehicle-treated controls. S-DPN treatment, however, had no significant effect on the open-arm exploration in βERKO mice, relative to the vehicle-treated control βERKO mice (Fig. 4, A and B).

ERβ receptor-mediated HPA axis reactivity after EPM is attenuated in the WT but not the βERKO mice

We examined the role of ERβ in HPA axis reactivity, by measuring plasma CORT and ACTH levels after the EPM, in WT and βERKO mice treated with S-DPN. S-DPN treatment resulted in a significant decrease in plasma CORT and ACTH levels in WT mice compared to the vehicle control ($P < 0.05$; Fig. 5, A and B), reflecting the decrease in anxiety-related behavioral response on the EPM. No such differences in CORT and ACTH levels were observed between the vehicle- and S-DPN-treated βERKO mice. Vehicle treatment demonstrated no significant difference in CORT and ACTH levels between the WT and βERKO mice.

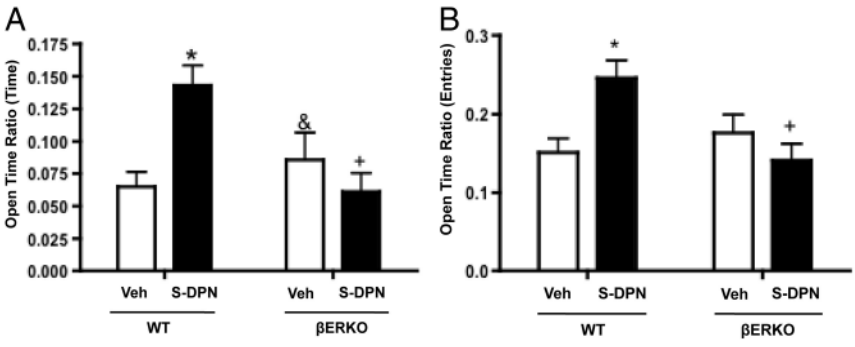

FIG. 4. Effect of S-DPN on anxiety-like behavior on the EPM. Ovariectomized WT and βERKO mice received daily injections of S-DPN or vehicle (Veh) for 7 d before behavioral assessment on the EPM. Both the genotypes displayed an increase in anxiety-like behavior, indicated by a reduction in open-arm exploration, measured as the OTR for time (A) and OTR for entries (B). S-DPN treatment significantly decreased both the parameters in WT mice (*, $P < 0.05$ compared with vehicle controls) but not in βERKO mice. Data are expressed as mean ± SEM, n = 9–12 per group. &, Significant difference ($P < 0.05$, compared with WT treated with S-DPN); +, significant difference ($P < 0.001$, compared with WT-treated with S-DPN).

ERβ receptor-mediated c-fos mRNA expression in the MEAad and BST, after EPM, is enhanced in the WT but not the βERKO mice

To study the cellular activity on the activation of the ERβ through S-DPN treatment, we measured the c-fos mRNA expression in neurons located in a variety of brain regions implicated in the modulation of anxiety-like behaviors. In response to the EPM, there was a significant increase in c-fos mRNA expression in the MEAad and BST after S-DPN treatment in the WT but not in the βERKO mice ($P < 0.05$; Fig. 6, A and B). No significant differences were found between the genotypes and treatments in any of the other brain regions examined, *i.e.* PVN, hippocampus, cingulated cortex, lateral septum, medial preoptic area, central amygdala, dorsomedial nucleus of hypothalamus, and arcuate nucleus (data not shown).

Discussion

Studies in rats and mice have demonstrated that the anxiolytic effects of estrogen are mediated through ERβ (12, 16). In the present study, using a combination of pharmacological and genetic approaches, we confirmed and extended the behavioral observations to elucidate the neural site(s) of ERβ action. Using the biologically active enantiomer, S-DPN, in βERKO mice, we demonstrated that the anxiety-like behavioral responses and HPA axis reactivity is attributable to ERβ. Furthermore, we identified MEAad and BST regions as the targets of ERβ action in the brain, by correlating the neural activation with increased c-fos mRNA expression.

Previous studies have demonstrated that DPN treatment significantly reduced anxiety-like behaviors in rats and mice (33–35). In the present study, we demonstrated that the peripheral administration of S-DPN decreases anxiety-like behaviors in ovariectomized, WT female mice, but not βERKO mice. We used three different behavioral assays, which are based on the animal's innate desire for the exploration of novel environments *vs.* the evasion of open and brightly lit areas. In the open field test, S-DPN administra-

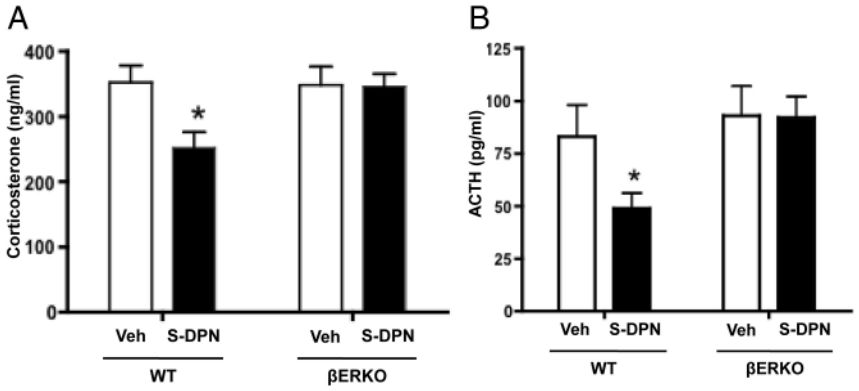

FIG. 5. Increases in plasma CORT (A) and ACTH (B) levels after the EPM are attenuated by S-DPN in WT mice. Plasma samples were collected within 10 min after the removal of animals from the EPM. Data are represented as mean ± SEM, n = 9–12 per group. *, Significant difference ($P < 0.05$) compared with vehicle-treated control.

tion decreased anxiety-like behaviors in the WT but not βERKO littermates. S-DPN-treated WT mice exhibited increased exploratory behavior in the center, spent more time in the center of the arena, and showed increased rearing behaviors (as measured by the vertical activity) compared to the βERKO littermates treated with S-DPN. In the light/dark exploration paradigm, S-DPN-treated WT mice displayed increased anxiolytic behaviors as compared to βERKO littermates, spending more time in the lit compartment than in the dark. Interestingly, no genotype × treatment interaction was evident in the number of transitions between the light and dark compartments. When tested in the EPM, the S-DPN treated WT mice, but not similarly treated βERKO mice, spent more time exploring and had more entries into the open arms of the maze. On the other hand, both, βERKO and the WT animals treated with vehicle did not display such anxiolytic behaviors. The current observations indicate that the anxiolytic effects of estradiol can be ascribed to ERβ activation as seen in WT mice but not in βERKO mice.

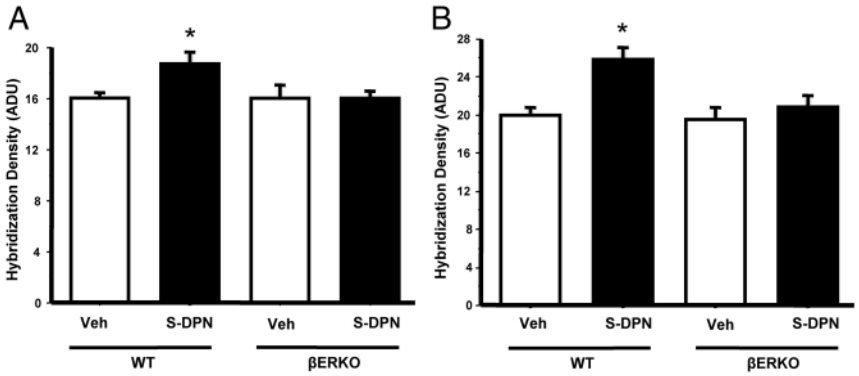

FIG. 6. S-DPN treatment increases c-fos mRNA expression in the MEAad (A) and BST (B) after the EPM test in the WT but not the βERKO mice. Relative levels of c-fos mRNA expression are shown. Data are represented as mean arbitrary density units (ADU) ± SEM, n = 9–12 per group. Statistically significant differences (*, $P < 0.05$) are observed in WT mice treated with S-DPN compared to the vehicle treatment.

In addition to the above-mentioned behavioral tests, another widely used behavioral assay to evaluate anxiety and depressive-related behaviors in rodents is the marble-burying task (36, 37). It is generally assumed that marble-burying behavior is an indicator of novelty-induced anxiety and active coping responses. Interestingly, in our studies on marble burying behavior, neither S-DPN nor vehicle had any significant effects in WT mice and their βERKO littermates. It could be interpreted that both the genotypes do not exhibit novelty-induced anxiety as reflected in marble burying. However, this explanation does not correlate with our findings on other anxiety-related assays such as open-field activity, light/dark exploration, and the EPM test, which consistently demonstrated that the WT mice exhibited anxiety-related behaviors that could be reduced by S-DPN treatment. In the absence of ERβ, S-DPN had no effect on the anxiety-like behaviors of βERKO mice. The other possibility is that the marble-burying behavior is not an anxiety-related response but is indicative of a repetitive and perseverative behavior (38, 39). In this context, it is interesting to note that both the genotypes do not exhibit defensive burying and do not have an obsessive-compulsive disorder component.

The HPA axis is the main neuroendocrine target for responses to stressful events, and anxiety disorders are frequently represented by dysfunction in this neuroendocrine system. After a stressful event, the stress hormones, CORT and ACTH, are up-regulated in the circulation. Gonadal steroids play an important role in modulating the HPA axis function. In female rodents, ovariectomy reduces stress-induced CORT and ACTH, which can be reversed by estradiol administration (4, 30). Activation of ERα by estradiol or an ERα selective agonist has been reported to increase neural activation in the PVN and potentiate CORT secretion (21, 40). Conversely, the activation of ERβ, using the ERβ selective agonists, racemic DPN, WAY-200070, and S-DPN, reduces stress-induced HPA axis reactivity as evidenced by a decrease in CORT and ACTH secretion (12, 19, 21). Furthermore, systemic administration or direct local administration of CORT into the central amygdala increases anxiety-like behaviors and HPA axis

reactivity (41, 42), an effect that is prevented by S-DPN administration (43).

Our results show ERβ mediates HPA axis reactivity because the peripheral administration of S-DPN reduces plasma CORT and ACTH levels in the WT but not in the βERKO mice after exposure to the EPM. These data suggest that ERβ's action is inhibitory to stress-induced hormone secretion. These observations are in concordance with the previously reported pharmacological studies of Lund *et al.* (12) in rats, which demonstrated that the nonselective ER antagonist, tamoxifen, blocked the inhibitory actions of DPN. Similar reductions in ACTH and CORT levels by DPN, in response to immobility stress, in rats have also been reported (44).

Estrogens have been shown to decrease c-fos mRNA expression, a marker of neural activation, in several limbic system nuclei in response to physiological stressors (45, 46). Conversely, local administration of estradiol into the PVN increases stress-induced c-fos mRNA expression (21, 40, 47). This dichotomous response has been assumed to be due to the region specific and selective activation of the two main ER subtypes. Although ERα activation by propylpyrazoletriol has been reported to augment neural activation of c-fos mRNA in the PVN after restraint stress for 30 min in rats, selective activation of ERβ by DPN significantly reduced c-fos mRNA expression in the PVN after the same stressor (40, 47).

In contrast to these observations in rats, S-DPN did not significantly alter mRNA expression levels of the immediate early gene c-fos in the PVN of WT mice and their βERKO littermates after the EPM. It is possible that this discrepancy could be due to the intensity of the stressor being used because studies by Lund *et al.* (40) and Weiser and Handa (47) used a robust restraint stress paradigm compared to the behavioral stressor (*i.e.* EPM) used in the current study. Indeed, several studies indicate that brain activation patterns of c-fos expression vary as a result of different stressors and vulnerability of animals to different types of stress (48–50). Interestingly, we observed a significant increase in EPM-induced levels of c-fos mRNA expression in the MEAad and BST after S-DPN treatment in the WT but not the βERKO mice. Activation of c-fos expression in the amygdala has previously been reported in rats exposed to the EPM (51). The absence of changes in c-fos mRNA expression in the PVN region by S-DPN in WT mice suggests the possibility that ERβ-mediated effects on parvocellular neurons of the PVN are not direct but indirect. This latter possibility is supported by the reduced HPA axis reactivity by the ERβ agonist in WT mice after the EPM. Taken together, the above data suggest that ERβ activates an inhibitory circuit within the amygdala and extended amygdala that can then elicit reduced activation of outputs regulating anxiogenic responses.

The precise mechanism for the ERβ regulation of HPA axis reactivity and of anxiety-related behaviors remains to be determined. A potential mechanism could involve oxytocin (OT), a neuropeptide that is synthesized by neurons in the PVN and supraoptic nucleus (52). Anxiety-related neuronal inputs are integrated at the level of PVN to induce secretion of corticotropin-releasing factor and arginine-vasopressin into the portal circulation. ERβ mRNA is highly expressed by neurons within the PVN (23, 53, 54), and a larger percentage colocalize with OT-containing neurons in magnocellular PVN of the rat, although lower levels have been reported in mice (21). Furthermore, ERβ is also found in arginine-vasopressin- and prolactin-expressing neurons (33–35, 54) and CRH-containing parvocellular neurons of the PVN (10–15%) (53, 54). This suggests that by binding to ERβ, estradiol could directly alter the function of PVN neuropeptide neurons. The observation that ERβ is found in almost all OT neurons of the PVN but not of the supraoptic nucleus, raises the interesting possibility that OT/ERβ-containing cells in the PVN may regulate HPA axis reactivity not only by local release of OT in the PVN but also by projections to extrahypothalamic areas, such as the MEAad, and BST, which can then feedback to inhibit HPA axis reactivity.

However, if OT's primary action occurs within the PVN, it is not necessarily a direct effect on the corticotropin-releasing factor-producing parvocellular neurons because the majority of neuronal responses to OT are excitatory (44), which would suggest an inhibitory intermediary. Thus, it appears that estradiol increases activity (c-fos mRNA expression) in the inhibitory interneurons, thereby increasing inhibitory tone and reducing anxiety-like behaviors and autonomic responses via ERβ.

In summary, using WT and βERKO mice in combination with the pharmacological tool, S-DPN, we demonstrate that estradiol's effects on anxiolytic behaviors and neuroendocrine responses are mediated by ERβ. In addition, we extended these studies to demonstrate the neuroanatomical sites of ERβ contribution to the estrogenic-regulation of stress response. The studies provide evidence that anxiolytic effects of estradiol could be mediated through ERβ-containing sites in brain regions, like the MEAad and the BST, which send projections to PVN, providing potential indirect mode of action.

Note Added in Proof

As this article was going to press, Carroll et al (55) have reported that their original speculation of S-DPN being the potent enantiomer [Sun *et al.* (20)] was incorrect. They

have currently reversed their prediction that R-enantiomer would be the preferred configuration and demonstrate that R-DPN is the more potent enantiomer. The designation of the more active enantiomer as S-DPN by Weiser *et al.* (22) was based on the earlier computational modeling- generated predictions of Sun *et al.* (20), not on absolute determination of this enantiomer's chemical structure. Carroll et al therefore suggest that the S-DPN used by Weiser et al. (22) was more likely to have been R-DPN. Since we used the same separation procedure as Weiser *et al.* (22) and followed their nomenclature, it is likely that the S-DPN used in the present study was also, in fact, R-DPN.

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