Novel Actions of Estrogen Receptor-β on Anxiety-Related Behaviors

Trent D. Lund, Tomislav Rovis, Wilson C. J. Chung, and Robert J. Handa

Estrogens are reported to have both anxiogenic and anxiolytic properties. This dichotomous neurobiological response to estrogens may be mediated by the existence of two distinct estrogen receptor (ER) systems, ERα and ERβ. In brain, ERα plays a critical role in regulating reproductive neuroendocrine function, whereas ERβ may be more important in regulating nonreproductive functions. To determine whether estrogen’s anxiolytic actions could be mediated by ERβ, we examined anxiety-related behaviors after treatment with ERα subtype-selective agonists. Ovariectomized female rats, divided into four treatment groups, were injected with the selective ERβ agonist diarylpropionitrile (DPN), the ERα-selective agonist propyl-pyrazole-triol (PPT), 17β-estradiol, or vehicle daily for 4 d. After injections, behavior was monitored in the elevated plus maze or open field. Rats treated with DPN showed significantly decreased anxiety-related behaviors in both behavioral paradigms. In the elevated plus maze, DPN significantly increased the number of open arm entries and time spent on the open arms of the maze. Furthermore, DPN significantly reduced, whereas PPT increased, anxiogenic behaviors such as the number of fecal boli and time spent grooming. In the open field, DPN-treated females made more rears, interacted more with a novel object, and spent more time in the middle of the open field than did control or PPT-treated rats. To confirm that DPN’s anxiolytic actions are ER mediated, the nonselective ER antagonist tamoxifen was administered alone or in combination with DPN. Tamoxifen blocked the previously identified anxiolytic actions of DPN. Taken together, these findings suggest that the anxiolytic properties of estrogens are ERβ mediated. (Endocrinology 146: 797–807, 2005)


**Materials and Methods**

**Animals and treatments**

Young (60- to 90-d-old) adult male and female Sprague Dawley rats were obtained from Charles Rivers Laboratories (Wilmington, MA) caged in pairs, housed in the Colorado State University vivarium, and maintained on a 12-h dark 12-h light schedule (lights on at 0700 h) with *ad libitum* access to food and water. One week after arrival, animals were gonadectomized under isoflurane anesthesia as previously described (23, 24).

**Hormone treatments**

One week after gonadectomy, animals were given a single daily sc injection for 4 d of either dimethylsulfoxide (DMSO; vehicle), 17-estradiol (E2; 0.25 mg/kg), DPN (1.0 mg/kg), or PPT (1.0 mg/kg) in a total volume of 0.2 ml. The doses of DPN and PPT used in these studies correspond to effective doses, relative to E2, that have been previously established and published (25, 26). PPT at 1 mg/kg had the same effect as an E2 dose of 0.1 mg/kg (26). One half hour after the final injection, animals underwent behavioral testing. Behavioral testing consisted of three noninvasive paradigms established as indicators of anxiety. Additionally, a subset of OVX females, treated as described above, were given concomitant injections of the nonselective ER antagonist tamoxifen [15.0 mg/kg, a dose known to effectively block ER in the rat brain (27, 28)]. Gonadectomized males were treated with vehicle or DPN and tested in only the elevated plus maze.

**DPN synthesis**

DPN was synthesized *de novo* as follows. To a homogeneous solution of sodium hydroxide (25 mmol) and tetrabutylammonium chloride (0.9 mmol) in water (10 ml) at 23 C, 4-(methoxyphenyl)-acetonitrile (60 mmol) and 4-(methoxybenzyl)-chloride (40 mmol) was added dropwise, the mixture was stirred for 16 h. The solvent was then removed in vacuo, the resulting purified by flash chromatography and/or recrystallization from the MeOH/CH2Cl2 mixtures. Purity of the resulting compound was checked with nuclear magnetic resonance. PPT was purchased from Tocris Cookson Inc. (Ellisville, MO); E2 was purchased from Sigma Chemical Co. (St. Louis, MO).

**Elevated plus maze**

Maze performance was evaluated as previously described (30, 31). The following measures were quantitated: (1) latency to enter the first arm; (2) the number of open and closed arm entries; (3) total time spent in open arms, closed arms and the center; (4) the total time spent grooming; (5) the number of fecal boli.

**Open field test**

The open field test was conducted as previously described (32). Measures scored in the test included 1) activity (total square crossings), 2) rearing, 3) grooming, 4) active sniffing, 5) gnawing, 6) head dips, and 7) number of fecal boli.

**Light/dark box**

Activity in the light/dark box was determined as previously described (33, 34). The following measures were scored: 1) initial latency to enter the dark compartment, 2) number of compartment entries, and 3) total time spent in each compartment. All behavioral testing was done between 0900 and 1200 h.

**Uterine weight**

Immediately after death, uteri were removed from the animal, dissected free of fat and connective tissue, and weighed.

**Plasma corticosterone analysis**

At killing, trunk blood was collected into ice-chilled tubes containing 0.5 m EDTA (200 ml) and 4 m g/ml aprotinin (100 ml). Blood was centrifuged and plasma removed and stored at −20 C until assayed for corticosterone via RIA as previously described (35, 36) using rabbit antichoristosterone serum (ICN Biomedicals, Inc., Costa Mesa, CA) at a final dilution of 1:2,000 according to manufacturer’s protocol. Standard curves were constructed from dilutions (5–500 ng/ml) of corticosterone (4-pregnen-11β, 21-diol-3, 20-dione; Steraloids, Wilton, NH).

**Progestosterone receptor (PR) immunocytochemistry**

PR expression in the medial preoptic nucleus (MPN) was visualized in a subset of females after hormone treatments using a polyclonal antibody raised in rabbit and directed against the DNA-binding domain (amino acids 533–547) of the human PR-A and PR-B (37, 38). Fresh-frozen cryostat sections (16 μm) through the MPN were 1) immersion fixed in 0.1 m phosphate buffer containing 5% acrolein for 2 h at room temperature (RT) and rinsed three times for 10 min each with Tris-buffered saline (TBS; 0.05 m Tris, 0.9% NaCl, pH 7.6), 2) treated with 0.1% NaBH4 made in TBS for 15 min, 3) rinsed three times for 10 min each with TBS, 4) treated with 0.3% H2O2 made in TBS for 15 min, 5) rinsed three times for 10 min each with TBS, 6) incubated with TBS containing 20% normal goat serum and 0.3% Triton X-100 (Sigma) for two times overnight at 4 C, 7) rinsed three times for 10 min each with TBS, 8) incubated with biotinylated goat antirabbit (Vector Laboratories, Burlingame, CA) 1:1000 diluted in TBS containing 2% normal goat serum and 0.3% Triton X-100 (Sigma) for 1 h at RT, 9) incubated with 1:1000 purified rabbit anti-PR polyclonal (Dako Corp., Glinston, Denmark) diluted with TBS containing 2% normal goat serum and 0.3% Triton X-100 (Sigma) for 1 h at RT, 10) rinsed three times for 10 min each with TBS, 11) incubated with avidin conjugated to peroxidase (Vector Laboratories, Burlingame, CA) 1:100 diluted in TBS containing 2% normal goat serum and 0.3% Triton X-100 for 60 min at RT, 12) rinsed three times for 10 min each with TBS, 13) reacted with 0.05% diazonium benzenediazide, 0.25% nickel ammonium sulfite, 0.01% H2O2 made in TBS for 10 min, 14) rinsed three times for 10 min each with TBS, and 15) dehydrated with increasing grades of alcohol and xylene and coverslipped using entellan (Merck, Darmstadt, Germany).
**Binding assay**

Cytosolic and nuclear ER concentration for hypothalamic preoptic area (HPOA), septum, and amygdala were determined by in vitro binding assay (35, 36). Protein was determined by the method of Lowry et al. (39), and DNA was determined by the method of Burton (40).

**Synthesis of hormone receptor proteins**

Full-length human ERα expression vector (pSG5-ERα; R. H. Price, University of California, San Francisco) and rat ERβ expression vector (pCDNA-ERβ; T. A. Brown, Pfizer, Groton, CT) were synthesized in vitro using the TnT-coupled reticulocyte lysate system (Promega, Madison, WI) with 17-RNA polymerase, during a 90-min reaction at 30°C. Translation reaction mixtures were stored at −80°C until used.

**Saturation isotherms**

To calculate and confirm the binding affinity of PPT and DPN for ER subtypes, 100-μl aliquots of reticulocyte lysate were incubated at optimal time and temperature, 90 min at room temperature (ERβ) and 18 h at 4°C (ERα), with increasing (0.01–50 nM) concentrations of [3H]E2. These times were determined empirically and represent optimal binding of receptor with E2. Nonspecific binding was assessed using a 200-fold excess of the ER agonist, diethylstilbestrol, in parallel tubes. After incubation, bound and unbound [3H]E2 were separated by passing the incubation reaction through a 1-ml lipophilic Sephadex LH-20 (Sigma) column according to previously published protocols (23, 41).

**Statistics and analysis**

Where appropriate, data were analyzed by ANOVA statistics followed by Newman-Keuls post hoc tests. Significance was set at P < 0.05. Curve fitting, scientific graphing, and analysis were completed using GraphPad Software (GraphPad Prism 3.0, San Diego, CA).

**Results**

**Screen of subtype-selective agonist binding**

Competition binding studies using in vitro translated ERs were conducted to calculate and confirm the ER binding properties and subtype preference of PPT and DPN. Our findings corroborate the previously published affinities and selectivity for both ERα and ERβ (20–22, 25, 26). Based on the ability of PPT to compete with [3H]E2 for ER binding, its selectivity for ER subtypes was confirmed, with PPT having a much higher affinity for ERα than for ERβ. In contrast, DPN bound with higher affinity to ERβ than to ERα. E2 had a similar affinity for both ERα and ERβ. A summary of these results is presented in Table 1.

**Peripherally administered DPN can bind ER in the brain**

PPT appears to cross the blood-brain barrier based upon its ability to up-regulate hypothalamic PR mRNA expression after peripheral administration (25). To determine whether peripherally administered DPN can also access the brain, we examined the concentration of occupied and unoccupied ER at several time points after DPN treatment using a standard in vitro binding assay (34, 35). This approach uses differential centrifugation to separate unoccupied receptor, which is found in the cytosolic fraction after high-speed centrifugation, from occupied receptor, which must be salt extracted from the cell nuclear fraction.

A single injection of DPN to OVX rats produced a significant increase in occupied, nuclear ER in the HPOA (one-way ANOVA F4,23 = 13.51; P < 0.0001) (Fig. 1A). This was accompanied by a stoichiometric decrease in unoccupied cytosolic ER (F4,23 = 8.01; P < 0.01) (Fig. 1B) in this same area compared with controls. Furthermore, both occupied and unoccupied receptors showed changes in level within 30 min (nuclear, P < 0.01; cytosolic, P < 0.01) after injection. This response was maintained (albeit decreased) at 6 h (occupied receptor, P < 0.01; unoccupied receptor, P < 0.01) and 12 h (occupied receptor, P < 0.01; unoccupied receptor, P < 0.01) after the injection. A similar pattern was identified in amygdala [occupied, F4,23 = 3.97, P < 0.01 (Fig. 1C); unoccupied, F4,23 = 6.52, P < 0.01 (Fig. 1D) and septum (occupied, F4,23 = 3.67, P < 0.01; unoccupied, F4,23 = 24.00, P < 0.01) ER (septum data not shown graphically). Based on these results, we calculate that the half-life of DPN binding in brain is 7.87 (± 1.03) h.

**DPN does not up-regulate PR immunoreactivity (PR-ir) in brain**

Using immunocytochemistry, we found that peripheral administration of E2 (0.25 mg/kg) and PPT (1 mg/kg) up-regulated PR-ir in the MPN of OVX females (Fig. 2, A–C). However, DPN treatment failed to increase PR-ir at the dose used in all of our studies (1 mg/kg body weight). PR-ir was indistinguishable between DPN-treated and DMSO-treated (Fig. 2A) females. Thus, because PR up-regulation seems to be an ERα-mediated event, the failure of DPN to up-regulate PR suggests strongly that DPN has no ERα activity at the dose used here.

**DPN is not uterotrophic**

Uterus weights differed significantly among OVX females treated sc once per day with DMSO (control) E2, PPT, and DPN (F1,28 = 12.81; P < 0.01) for 4 d. Treatments of E2 and PPT caused a significant increase in weight compared with controls [E2, 0.631 ± 0.014 g (mean ± SEM); PPT, 0.628 ± 0.063 g; control, 0.287 ± 0.014 g], whereas DPN (0.222 ± 0.010 g) treatment did not differ from controls.

**DPN treatment decreases fear and anxiety behavior**

To test our hypothesis that ER subtypes play differing but crucial roles in mediating fear and anxiety behaviors, we tested animals in three behavioral paradigms: the elevated plus maze, the open field test, and the light/dark box (a different set of animals was used for each paradigm).

**Elevated plus maze.** In the elevated plus maze, rodents avoid the open, elevated, and brightly lit arms of the maze and prefer to remain in the more darkly lit, closed arms. A re-
duction in anxiety is indicated by an animal’s tendency to spend more time interacting with their environment through exploration (rearing and head dips) of the maze that leads to their entering and spending more time in the open arms of the maze.

When tested in the elevated plus maze, OVX females...
treated with DPN displayed significantly fewer anxiety-related behaviors. This was evidenced by increased numbers of entries onto the open arms (F3,31 = 9.58; P < 0.01) (Fig. 3A) and increased time spent on the open arms (F3,31 = 19.75; P < 0.01) (Fig. 3B) compared with control and PPT-treated animals. DPN-treated females also displayed more rearing behavior (F3,31 = 6.23; P < 0.01) (Fig. 3C) and more head dips (F3,31 = 6.73; P < 0.01) (Fig. 3D) compared with control and PPT-treated females. Furthermore, DPN significantly reduced, whereas PPT increased, anxiogenic behaviors such as the number of fecal boli (F3,31 = 15.34; P < 0.01) (Fig. 3E) and time spent grooming (F3,31 = 7.52; P < 0.01) (Fig. 3F) compared with control females. Consistent with this, plasma samples collected one half hour after removal from the maze showed that corticosterone levels were significantly reduced in DPN-treated animals and significantly increased in E2- and PPT-treated animals (F3,30 = 12.63; P < 0.01) (Fig. 4) relative to control females.

All animals tested in the maze had significantly higher plasma corticosterone levels compared with nonstressed controls (F1,30 = 555; P < 0.01) (Fig. 4) killed directly from their home cages. Nonstress corticosterone levels did not differ among treatments.

---

Fig. 3. DPN treatment of female rats reduced anxiety-related behavior in the elevated plus maze. The following behaviors were quantitated: open arm entries (A), time spent on the open arms (B), rears (C), head dips (D), fecal boli (E), and time spent grooming (F) after four daily injections of DPN, PPT, E2, or vehicle. n = 9 animals per treatment group; for PPT, n = 8. *, Significant difference (P < 0.01) compared with control treatment.
Open field test. DPN treatment reduced anxiety-related behavior in the open field paradigm in a fashion similar to that observed in the elevated plus maze. In the open field, measure of time in the center of the arena vs. time in the perimeter gives a measure of anxiety-related behaviors. In a brightly lit open area, rats tend to stay near the walls of the open field and avoid the center. Less anxious animals spend more time engaging in exploratory behaviors as evidenced by increased time in the center, increased rearing, and increased encounters with novel objects.

DPN- and E2-treated females exhibited more rearing ($F_{3,20} = 9.71; P < 0.01$) (Fig. 5A) and more interaction with a novel object ($F_{3,20} = 5.03; P < 0.01$) (Fig. 5B) and spent more time in the middle of the maze of the open field ($F_{3,20} = 7.30; P < 0.01$) (Fig. 5C) than did control or PPT-treated females. However, in the assessment of general locomotor activity, PPT-treated females engaged in significantly more activity (total square crossings, $F_{3,20} = 7.83; P < 0.01$) (Fig. 5D) compared with all other treatment groups.

**FIG. 4.** DPN decreases plasma levels of corticosterone. Plasma corticosterone levels were determined from plasma samples taken one half hour after removal from the maze. Nonstressed controls were killed directly from their home cages. n = 5 animals per treatment group. *, Significant difference ($P < 0.01$) compared with control treatment 30 min after stress.

**FIG. 5.** DPN treatment of female rats reduced anxiety-related behavior in the novel open field. Animals were tested in a novel open field after four daily injections of DPN, PPT, E2, or vehicle. The number of rears (A), time spent exploring a novel item (B), time in the middle squares (C), and overall locomotor activity (total square crossings (D) were quantitated. n = 6 animals per treatment group. *, Significant difference ($P < 0.01$) compared with control treatment.
Light/dark box. Consistent with our results above, OVX female rats treated with DPN showed significantly less anxious behavior in the light/dark box. The light/dark exploration task represents a naturalistic conflict between the tendencies of rodents to explore a novel environment vs. the tendency of rodents to avoid the brightly lit open area. DPN-treated females spent more time in ($F_{1,8} = 27.57; P < 0.01$) and made more transitions to ($F_{1,8} = 10.00; P < 0.01$) to the lit compartment than did control females (data not presented graphically).

**DPN's anxiolytic actions are ER mediated**

To establish that DPN's anxiolytic actions are ER mediated, we administered the nonselective ER antagonist tamoxifen alone or in combination with DPN or E2 to OVX females. In this study, we replicated our finding that DPN enhanced anxiolytic behaviors and also found that tamoxifen blocked the anxiolytic actions of both E2 and DPN. DPN and E2-treated females made more entries onto ($F_{5,24} = 4.14; P < 0.01$) (Fig. 6A) and spent more time on ($F_{5,24} = 5.86; P < 0.01$) (Fig. 6B) the open arms than control or tamoxifen-alone treated groups. Concomitant treatment of E2 or DPN with tamoxifen prevented these effects. Furthermore, the DPN-treated females displayed more head dips ($F_{5,24} = 5.17; P < 0.01$) (Fig. 6C) and fewer fecal boli ($F_{5,24} = 2.50; P < 0.01$) (Fig. 6D). Again, these effects were blocked by concomitant treatment with tamoxifen.

**DPN treatment decreases fear and anxiety behavior in male rats**

To determine whether ERβ regulates anxiety-related behaviors in gonadectomized male rats, similar to that observed in OVX females, we examined the ability of DPN to alter the behavior of 75-d-old adult male rat's performance in the elevated plus maze. Gonadectomized males treated with DPN made significantly more entries onto the open arms ($F_{1,14} = 5.03; P < 0.05$) (Fig. 7A) and spent more time on the open arms ($F_{1,14} = 6.22; P < 0.05$) (Fig. 7B) than did...
vehicle-treated males. DPN-treated males also displayed more rearing behavior (F1,14 = 30.37; P < 0.01) (Fig. 7C), more head dips (F1,14 = 5.09; P < 0.05) (Fig. 7D), and less grooming behavior (F1,14 = 11.07; P < 0.01) and exhibited a trend toward a decrease in the number of fecal boli (F1,14 = 1.94; P = 0.10) compared with vehicle-treated males.

Discussion

The results from the present study support the hypothesis that estrogen’s dichotomous action in regulating mood and anxiety-related behaviors are mediated by the opposing and distinct roles of ERα and ERβ. Our data show that the ERβ-subtype-selective agonist DPN can cross the blood-brain barrier and occupy neural ERs with a half-life of approximately 8 h. In doing so, it is able to inhibit anxiogenic and enhance anxiolytic behaviors on three different tasks. These actions of DPN are blocked by concomitant treatment with tamoxifen, indicating that this is truly an ER-mediated event.

These studies used recently developed ER-subtype-selective agonists rather than knockout models to directly test the hypothesis that either ERα or ERβ is specifically involved in altering anxiety-related behaviors. Although a valuable resource, the use of knockout mouse models is of limited utility to address the present question. The absence of one receptor subtype may result in unrecognized differences in brain ontogeny or the potential development of compensatory mechanisms, or altered hormonal profiles (42, 43) may complicate the interpretation of data resulting from their use. Nonetheless, our data are largely consistent with that of Krezel et al. (17), who showed that female ERβ knockout mice have an increase in anxiogenic behaviors in the elevated plus maze. In contrast, Krezel et al. (17) did not find that male ERβ knockout mice had increased anxiogenic behaviors. However, our results clearly show that male rats can respond to DPN with an increase in anxiolytic behaviors, suggesting that ERβ is not sex specific in its anxiolytic actions. The differences between our results and those of Krezel et al. (17) may be because of the presence vs. absence (our study) of testes, species differences, or the inherent differences between studying knockout models vs. wild-type animals.

DPN crosses the blood-brain barrier

Although PPT has been shown to have neural effects (e.g. the induction of PR mRNA) after systemic administration in
vivo (25), it has not been previously shown whether peripherally administered DPN could effectively cross the blood-brain barrier. Our data show that within 30 min after peripheral administered DPN, increases in salt-extractable nuclear ER binding are detectable in brain, thus indicating that DPN accesses the brain. Increases in nuclear ER are accompanied by a stoichiometric decrease in unoccupied receptor in the cytosolic fraction of HPOA, amygdala, and septum, further indicating that these changes are not because of alterations in the total numbers of receptor, but rather because of activation of the existing receptor population. In all brain regions sampled, both occupied and unoccupied receptors showed changes within 30 min of the injection, a response that was maintained up to 12 h after the injection. These brain areas were chosen for sampling because ERβ mRNA and protein are reportedly expressed at high levels within these brain areas (44, 45), and nuclei within these areas have been associated with fear and anxiety-related behaviors (18, 19).

DPN does not activate ERα in brain

Notwithstanding DPN’s effectiveness in crossing the blood-brain barrier and binding ERs, DPN, at the dose given in these studies (1 mg/kg), did not up-regulate PR-ir in the MPN. In contrast, we show that PPT can effectively increase PR-ir, which confirms previous findings identifying PPT’s ability to induce PR mRNA (25). Furthermore, because both PPT and E2, but not DPN, up-regulate PR-ir, this strongly suggests that PR-ir in the MPN is ERα mediated. Such findings also indicate that DPN does not activate ERα at the dose used here. In light of DPN’s ability to bind ER in brain, our data suggest that DPN’s action on behaviors is ERβ mediated. Additionally, DPN, unlike E2 and PPT, did not alter uterine weights, suggesting that the dose of DPN given did not activate ERα peripherally.

ERβ mediates anxiety-related behavior

Peripheral administration of the selective ERβ agonist DPN decreased anxiety-related behaviors in three different behavioral paradigms based on the natural conflict between their desires to explore a novel environment vs. avoidance of the brightly lit arena. In the light/dark box paradigm, DPN-treated females displayed less anxious behavior than control animals, spending more time on and making more transitions into the lit compartment. In the open field paradigm, similar observations were made such that both E2 and DPN decreased anxiety behavior. DPN- and E2-treated females spent more time in the middle, open squares and engaged with a novel object and exhibited more rearing behaviors than control females and females treated with the ERα-selective ligand PPT. It is likely that this increased exploratory behavior by E2- and DPN-treated females was responsible for the observed decrease in general locomotor activity. Because PPT-treated females engaged in less novel object exploration and rearing behavior than control or E2- or DPN-treated females, it follows that more of their time would be spent in general activity assessed as number of square crossings. It is, therefore, unlikely that the observed anxiolytic effects of DPN can be attributed solely to a general increase in locomotor activity.

In the elevated plus maze, when an additional anxiety-producing component was introduced by elevating the maze 5 ft above the ground, DPN- and E2-treated females had decreased levels of anxiety. Females treated with DPN had an increased number of rears and head dips and made more entries onto the open arms and subsequently spent more time on the open arms of the maze than PPT-treated and control animals. Additionally, the behavior of E2-treated females, in spending significantly more time on the open arms of the maze than control or PPT-treated females, also suggests a decrease in anxiety in E2-treated females relative to control and PPT-treated females. Furthermore, the anxiolytic actions of DPN are not limited to females. Males treated with DPN displayed far less anxiety-related behavior compared with controls. DPN-treated males relative to controls made more arm entries, spent more time on the open arms, and had increased rearing and head dips and decreased grooming and fecal boli. In contrast to DPN and in comparison with control and E2, OVX females treated with PPT displayed some increased anxiogenic behaviors. Although these behaviors, number of fecal boli, and time spent grooming are well established indicators of increased anxiety (30, 31, 46), PPT’s anxiogenic effect may not have manifested itself behaviorally in other measures because of floor effects in the test paradigm.

DPN’s anxiolytic actions are ER mediated

The possibility exists that DPN’s actions as an anxiolytic may not be entirely ERβ mediated. For instance, it is possible that DPN may act in a manner similar to the benzodiazepines to reinforce transmission at GABA(A) receptors, or as serotonin (5-HT)(1A) receptor agonists and 5-HT reuptake inhibitors similar to the principal drugs currently employed in the management of anxiety disorders. In support of this, 5-HT-containing neurons in the raphe nucleus have been shown to contain ERβ (47–49), and GABA-containing interneurons also appear to be ERβ positive (50, 51). Nonetheless, the ability of tamoxifen to block DPN’s anxiolytic actions strongly suggests that an ER is mediating its effects. Although there are compounds that are superior to tamoxifen (i.e. ICI 182,780) in their ability to act as pure antagonists of ER, these compounds are incapable of crossing the blood-brain barrier. Unfortunately, to our knowledge there exists no available ERβ-selective antagonist at the present time. Therefore, because it was critical that we block ER in brain, we were limited in these studies to the use of tamoxifen. In all treatment paradigms, tamoxifen had no effect of its own.

It is also highly likely that DPN’s actions are because of binding of ERβ and not because of nonselective interactions with ERα. Although a recent publication suggests that a lower dose of DPN could also work to regulate mood (52), the higher dose used in these studies appears to be acting solely via ERβ. The ERα-selective agonist did not mimic the actions of DPN and often had effects opposite that of DPN. Furthermore, DPN’s actions were different qualitatively and quantitatively from the mixed agonist E2. Moreover, DPN
was ineffective in inducing PR expression in the MPN. Taken together, the above findings support the hypothesis that ERβ is a critical factor in the regulation of anxiety-related behaviors and that in some cases ERα and ERβ may work in opposition to provide precise control of such behaviors.

**DPN decreases plasma levels of corticosterone**

After physical or psychological stressors, females typically display a more robust hormonal response than males (53–55). It appears that in males, androgens act to inhibit (53–55), whereas in females, estrogens function to enhance (35, 36, 53) the activity of the hypothalamo-pituitary adrenal axis. Endocrine manipulation studies in females show that OVX can reduce the stress-induced secretion of corticosterone, a reduction that is reversed via E2 administration (35, 36, 53). In the present study, 30 min after testing in the elevated plus maze, corticosterone levels were increased in females treated with E2 and PPT treatments but decreased by DPN-treated animals relative to OVX controls. This finding suggests that the aforementioned increase in circulating levels of corticosterone is a function of ERα activation and that ERβ’s action is consistently opposite that of ERα and inhibitory to stress-induced hormone secretion. Because DPN decreases circulating levels of corticosterone, the possibility does exist that DPN’s anxiolytic effects are caused at least in part by regulation of adrenal secretions, or the result could be epiphenomenal to the reduced anxiety in these animals. Research has identified that the anxiolytic action of other compounds (isapipran and buspirone) are prevented by adrenalectomy (14), making this a viable explanation of DPN’s mechanism of action. Studies examining such an interaction are currently underway.

In summary, activation of ERβ by the selective agonist DPN decreases anxiety-related behavior. Our results demonstrate that DPN crosses the blood-brain barrier to have its effect on behaviors. Furthermore, DPN decreases anxiety-related behavior in both sexes and across a variety of behavioral paradigms. Because the anxiolytic effects of DPN are extinguished by the ER antagonist tamoxifen, DPN’s effects are likely ER mediated. Moreover, based upon DPN’s selectivity to ERβ, it is ERβ that mediates the anxiolytic effects of estrogens. Thus, selectively targeting neural ERβ activation in women may be an important factor to consider in the design of future therapeutics for anxiety and depression.

**Acknowledgments**

We thank D. Munson, J. Evans, M. McNulty, and D. Moore for their expert technical assistance.

Received August 31, 2004. Accepted October 19, 2004.

Address all correspondence and requests for reprints to: Trent D. Lund, Ph.D., Department of Biomedical Science, Colorado State University, Anatomy W103, 1617 Campus Delivery, Fort Collins, Colorado 80523-1670. E-mail: tlund@colostate.edu.

This work was supported by the National Institutes of Health (NIH R01 NS039951).

**References**


31. Landgraf R, Wigger A, Holshofer F, Neumann ID 1999 Hyper-reactive hy-
32. Handa RJ, Cross MK, George M, Gordon BH, Burgess LH, Cabrera TM, Hata
N, Campbell DB, Lorenz SA 1993 Neuroendocrine and neurochemical re-
sponses to novelty stress in young and old male F344 rats: effects of d-
33. Crawley JN 1999 Behavioral phenotyping of transgenic and knockout mice: ex-
perimental design and evaluation of general health, sensory functions, mo-
tor abilities, and specific behavioral tests. Brain Res 835:18–26
34. Crawley JN, Paylor RA 1997 Proposed test battery and constellations of specific behavioral paradigms to investigate the behavioral phenotypes of transgenic and knockout mice. Horm Behav 31:197–211
35. Burgess LH, Handa RJ 1992 Chronic estrogen-induced alterations in adreno-
corticotropin and corticosterone secretion, and glucocorticoid receptor-medi-
ated functions in female rats. Endocrinology 131:1261–1269
P 1990 Two distinct estrogen-regulated promoters generate transcripts encod-
ing the two functionally different human progesterone receptor forms A and
B. EMBO J 9:1603–1614
38. Trash AM, Wotiz HH 1990 Monoclonal and polyclonal antibodies to human
progesterone receptor peptide-(533–547) recognize a specific site in unacti-
vated (8S) and activated (4S) progesterone receptor and distinguish between intact and proteolyzed receptors. Endocrinology 127:1167–1175
39. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ 1951 Protein determination
41. O’Keefe JA, Handa RJ 1990 Transient elevation of estrogen receptors in the
JA 2000 Estrogen receptor (ERβ), a modulator of ERα in the uterus. Proc Natl
Acad Sci USA 97:9936–9941
44. Shughrue PJ, Lane MV, Merchenthaler I 1997 Comparative distribution of estrogen receptor-α and -β mRNA in the rat central nervous system. J Comp
Neurol 386:507–525
45. Shughrue PJ, Merchenthaler I 2001 Distribution of estrogen receptor β im-
umnoreactivity in the rat central nervous system. J Comp Neurol 436:64–81
47. Lu H, Ozawa H, Nishi M, Ito T, Kawata M 2001 Serotonergic neurones in the
dorsal raphe nucleus that project into the medial preoptic area contain oes-
48. Gundlah C, Lu NZ, Mirkes SJ, Beetha CL 2001 Estrogen receptor β (ERβ)
mRNA and protein in serotonin neurons of macaques. Brain Res Mol Brain Res 91:14–22
49. Alves SE, Weiland NG, Hayashi S, McEwen BS 1998 Immunocytochemical
localization of nuclear estrogen receptors and progesterin receptors within the
50. Blurton-Jones M, Tusznynski MH 2002 Estrogen receptor-β colocalizes exten-
sively with parvalbumin-labeled inhibitory neurons in the cortex, amygdala, 
basal forebrain, and hippocampal formation of intact and ovariectomized adult
52. Walf AA, Rhodes ME, Frey CA 2004 Antidepressant effects of ERβ-selective
estrogen receptor modulators in the forced swim test. Pharmacol Biochem
Behav 78:523–529
hypothalamo-pituitary-adrenal axis: novel roles for androgen and estrogen
receptors. In: Recent research developments in endocrinology. Kerala, India:
Transworld Research Network; 69–86
54. Gaskin JH, Kitay J 1970 Adrenocortical function in the hamster: sex differ-
ces and effects of gonadal hormones. Endocrinology 87:779–786
55. Handa RJ, Nunley KM, Lorenz SA, Louie JP, McGivern RF, Bollnow MR
1994 Androgen regulation of adrenocorticotropic and corticosterone secretion in the male rat following novelty and foot shock stressors. Physiol Behav 55:117–124

Endocrinology is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.