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Estrogen Receptor β, but not α, Mediates Estrogen's Effect on Cocaine-Induced Reinstatement of Extinguished Cocaine-Seeking Behavior in Ovariectomized Female Rats

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Preclinical and clinical studies indicate that females are more vulnerable to relapse than males, and the neurobiological effects of estrogen are thought to mediate, in part, the sex differences in cocaine-taking behavior. The goal of the present study was to investigate the involvement of estrogen receptor α (ERα) and β (ERβ) in estrogen-mediated increases in cocaine-induced reinstatement of extinguished cocaine-seeking behavior in ovariectomized (OVX) female rats. Rats were initially trained to self-administer cocaine (0.4 mg/kg/inf, i.v.) under a fixed-ratio 1 (FR 1) schedule of reinforcement during daily 2-h sessions. After a 10-day maintenance period, cocaine solutions were replaced with saline, and self-administration was extinguished over a 14-day period. OVX rats were then treated with either the mixed ERα/β agonist estradiol benzoate (EB), the ERα-selective agonist, propyl-pyrazole-triol (PPT), the ERβ-selective agonist, diarylpropionitrile (DPN), or a vehicle control (dimethyl sulfoxide, DMSO). Treatment lasted a total of 9 days, and during this time, rats were assessed for nonreinforced reinstatement of extinguished cocaine-seeking behavior after priming injections of saline or cocaine (5, 10, or 15 mg/kg, i.p.). OVX rats showed no differences in self-administration during maintenance or extinction. OVX rats treated with EB exhibited greater responding for cocaine during reinstatement compared to OVX + DMSO controls. Selective activation of ERβ with DPN also increased cocaine-induced reinstatement responding, whereas selective activation of ERα with PPT did not affect cocaine-seeking behavior. These results indicate that estrogen influences the propensity for reinstatement of extinguished cocaine-seeking behavior, and that estrogen-mediated enhancement of cocaine-induced reinstatement responding involves the activation of ERβ.
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INTRODUCTION

Studies in both humans and laboratory animals suggest that females are more vulnerable to drug abuse and addiction than males. It has been reported that females are more responsive than males to stimuli that cause relapse, including exposure to stress (Klein *et al*, 1997), drugs (Lynch and Carroll, 2000; Kippin *et al*, 2005), and drug-associated cues (Robbins *et al*, 1999; Elman *et al*, 2001, but see also Avants *et al*, 1995; Fuchs *et al*, 2005). Research on sex differences in the behavioral and neurochemical responses to drugs, particularly the stimulant drugs cocaine and amphetamine/methamphetamine, indicates that the gonadal hormone estrogen may be responsible for the enhanced drug-induced responses in females compared to males (for review, see Becker, 1999; Festa and Quiñones-Jenab, 2004). Self-administration studies suggest that estrogen also enhances drug reward and/or the motivation for drug-seeking behavior (for review, see Lynch *et al*, 2002; Carroll *et al*, 2004; Roth *et al*, 2004). For example, it has been shown that removal of endogenous estrogen by ovariectomy (OVX) decreases acquisition of cocaine self-administration (Lynch *et al*, 2001) and cocaine-primed reinstatement of extinguished cocaine-seeking behavior in rats (Larson *et al*, 2005). Conversely, exogenous estrogen administration in OVX rats increases acquisition of cocaine self-administration (Lynch *et al*, 2001; Hu *et al*, 2004; Jackson *et al*, 2006) and cocaine-induced reinstatement of cocaine-seeking behavior (Larson *et al*, 2005).

Given the high rates of relapse to cocaine use, and the finding that females may be more likely than males to relapse, it is important to understand the mechanisms underlying estrogen's effects on cocaine-seeking behavior. Estrogen is known to produce many of its effects by acting at its major estrogen receptor (ER) subtypes, ERα and ERβ. Both ERα and ERβ are highly expressed in the central

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nervous system, particularly in the brain in limbic-associated structures such as the amygdala, the hypothalamus, and the bed nucleus of stria terminalis. However, the concentration and distribution of each receptor subtype within as well as outside these regions can vary greatly (Laflamme *et al*, 1998, reviewed by McEwen and Alves, 1999). It has been reported that ERβ mRNA exists in brain regions where there is little or no ERα (eg, isocortex, cerebellum, ventral tegmental area (VTA)), and ERα can be found in other regions (eg, periaquaductal grey, arcuate nucleus, subfornical organ) that have little or no ERβ (Kuiper *et al*, 1997; Shughrue *et al*, 1997; Laflamme *et al*, 1998). However, it should be noted that the relative abundance of ERα and ERβ found in various brain regions can be ambiguous. For example, whereas a predominance of ERβ mRNA has been consistently reported throughout the isocortex (Laflamme *et al*, 1998; Shughrue *et al*, 1997), there is conflict regarding which ER subtype predominates in the allocortex (Laflamme *et al*, 1998; Shughrue *et al*, 1997). Similarly, variable results have been found regarding ER localization in the substantia nigra, with some investigators finding more ERα than ERβ mRNA in the pars compacta (Laflamme *et al*, 1998), and others reporting only weak staining for ERβ mRNA in this region (Shughrue *et al*, 1997).

In some brain areas such as the striatum, estrogen effects are well documented (for review, see Becker 1999); however, the existence of either receptor subtype in these areas is controversial (Pfaff and Keiner, 1973; Roy *et al*, 1990; Shughrue *et al*, 1997; Kuppers and Beyer, 1999). For example, one study found no ERα or ERβ mRNA in the striatum of adult male and female rats (Laflamme *et al*, 1998), whereas another study found both ERα and ERβ mRNA in the striatum of adult mice (Kuppers and Beyer, 1999). Also, in the nucleus accumbens (ventral striatum) of adult rats, ERβ mRNA labeling has been reported (Shughrue *et al*, 1997), but only minimal levels of ERβ protein have been found (Shughrue and Merchenthaler, 2001). Nevertheless, given the important role estrogen seems to play in drug abuse and the finding that ERs exist in many of the brain regions implicated in the rewarding and motivational aspects of drugs of abuse (eg, VTA, amygdala, nucleus accumbens, and cortex), further investigation of the role of ERα and ERβ on cocaine-seeking behavior in rats in warranted.

In order to better understand the functions of ERα and ERβ, researchers have typically taken advantage of the availability of ER selective knockouts (ERKOs) and selective ER modulators (SERMs) such as ER selective agonists (Stauffer *et al*, 2000; Meyers *et al*, 2001) and antagonists (Barkhem *et al*, 1998; Hall *et al*, 2000; Sun *et al*, 2005). Use of these techniques have helped to identify the involvement of ERα and/or ERβ in several biological functions, including mating and reproduction (Ogawa *et al*, 1998, 1999; Hewitt and Korach, 2003), anxiety (Krezel *et al*, 2001; Walf and Frye, 2005; Imwalle *et al*, 2005; Lund *et al*, 2005), learning and memory (Fugger *et al*, 2000; Rissman *et al*, 2002), wheel running (Ogawa *et al*, 2003), and food intake (Geary *et al*, 2001; Roesch, 2006). For example, ERKO studies in mice have shown that ERα is required for normal sexual receptivity and fertility in mice and rats, whereas ERβ does not appear to be critical for reproductive function (Hewitt and Korach, 2003). Studies in rats using SERMs have demonstrated that activation of ERβ increases, whereas activation of ERα decreases or has no effect on, the number of entries or time spent in the open arms of an elevated plus maze, indicating that ERβ mediates the anxiolytic effects of estrogen (Lund *et al*, 2005; Walf and Frye, 2005). Furthermore, studies using both techniques have suggested that ERα and ERβ may both be important for hippocampal-dependent learning and memory, although results can vary depending on the task (eg, inhibitory avoidance and spatial learning, and so on) being measured (Fugger *et al*, 2000; Rissman *et al*, 2002).

Although several studies have examined estrogen-mediated effects on cocaine-mediated responses (Lynch *et al*, 2002; Festa and Quiñones-Jenab, 2004; Roth *et al*, 2004), including the reinstatement of cocaine-seeking behavior after an abstinence period (Fuchs *et al*, 2005; Larson *et al*, 2005; Kippin *et al*, 2005), no studies to date have investigated the role of ERα and ERβ in these effects. Therefore, the goal of the present study was use ER selective agonists to identify the involvement of ERα and ERβ in estrogen's ability to enhance reinstatement of cocaine-seeking behavior in female OVX rats. Four groups of OVX rats were initially allowed to self-administer cocaine by lever-press, and they were subsequently exposed to 14 days of extinction (abstinence). At the end of the extinction phase, each group received one of the following treatments: (1) 17-β estradiol benzoate (EB), a mixed ERα/β agonist, (2) propyl-pyrazole-triol (PPT), a selective ERα agonist with a 410-fold selectivity for ERα over ERβ (Stauffer *et al*, 2000), (3) diarylpropionitrile (DPN), a selective ERβ agonist with a 70-fold selectivity for ERβ over ERα (Meyers *et al*, 2001), or (4) the vehicle, dimethyl sulfoxide (DMSO, control). Three days after the start of treatment, rats were exposed to a reinstatement protocol in which saline or cocaine priming injections were given in an alternating manner, and responding on the previously active (cocaine paired) and inactive (not cocaine paired) levers was measured, but not reinforced. Reinstatement of responding on the previously active lever was considered an indicator of cocaine-seeking behavior, and responding was compared between groups across a range of cocaine doses.

MATERIALS AND METHODS

Subjects

A total of 41 experimentally naïve, adult (mean age at surgery: 86.2 ± 2.78 days) female Wistar rats were used as subjects in this study ($n = 10$–11 per group). Rats were obtained from Harlan Sprague–Dawley (Madison, WI) and they were pair-housed in plastic holding cages before surgery. During this time, rats were given *ad libitum* access to food (pellet chow, Purina Mills, Minneapolis, MN) and water. After surgery, they were individually housed in their experimental chambers, where they remained for the duration of the experiment. Intake of food (ground pellet chow, Purina Mills, Minneapolis, MN) and water were measured every morning (0800–0900 h). Each group of rats was fed a fixed amount of food (16 g) every afternoon (1500 h) to equalize food intake. All laboratory rooms where rats were housed were kept under controlled temperature

(21–23°C), humidity, and light (12/12 light/dark cycle, lights on at 0600) conditions. The University of Minnesota Institutional Care and Use Committee (IACUC) approved the experimental protocol under protocol number 0410A64760, and experimental procedures conformed to the Principles of Laboratory Animal Care (National Research Council, 2003). The Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) approved and accredited all laboratory facilities.

Drugs

Cocaine (NIDA, Research Triangle Park, NC) was dissolved in a sterile 0.9% saline solution containing heparin (5 USP U/ml). Cocaine stock solutions were made every 2 weeks, and they were kept refrigerated before use, at which time they were added to syringe pumps (for self-administration) or 1 ml syringes (for reinstatement testing) that were then brought to room temperature. The cocaine solution (1.6 mg/ml) that was used for self-administration (0.4 mg/kg/inf, i.v.) was delivered at a rate of 0.025 ml/s and a duration of 1 s/100 g body weight. For reinstatement, cocaine (8.0 mg/ml) or saline vehicle was prepared such that each rat received the same injection volume on each testing day regardless of the cocaine dose (0, 5, 10, or 15 mg/kg, i.p.). The DMSO vehicle and EB were purchased from Sigma-Aldrich (St Louis, MO), and PPT and DPN were obtained from Tocris Bioscience (Ellisville, MO). All treatment drugs were dissolved in DMSO, and the concentrations for all estrogen agonist treatments (1.0 mg/ml EB, 1.0 mg/ml PPT, and 1.0 mg/ml DPN) were prepared so that each rat received similar injection volumes during treatment. The dose of EB (0.05 mg/kg/inj, subcutaneously (s.c.)) was based on what has been previously shown to enhance cocaine-induced reinstatement of lever responding in OVX rats (Larson *et al*, 2005). The doses of PPT (1.0 mg/kg/inj, i.p.) and DPN (1.0 mg/kg/inj, i.p.) correspond to effective doses that have been previously reported (Harris *et al*, 2002; Frasor *et al*, 2003; Lund *et al*, 2005; Le Saux and Di Paolo, 2005; Le Saux *et al*, 2006). These previous reports indicate that, in some cases, selective activation of ERα or ERβ by a 1.0 mg/kg dose of PPT (ERα) or DPN (ERβ) produced similar effects, such as in the striatum, where both increased preproenkephalin mRNA levels (Le Saux and Di Paolo, 2005). Other times, selective activation of ERα and ERβ had opposing effects. For example, a 1.0 mg/kg dose of PPT decreased the time spent in the open arms of an elevated plus maze, whereas the same dose of DPN increased it compared to controls, suggesting opposing influences of ERα and ERβ on anxiety behavior (Lund *et al*, 2005). In other cases, only activation of one of the ER subtypes had an effect, as shown by the ability of a 1.0 mg/kg dose of DPN, but not PPT, to increase D_2 receptor levels in the striatum and nucleus accumbens (Le Saux *et al*, 2006).

Apparatus

Custom-made operant chambers were used for housing rats after surgery, and rats lived in these chambers for the duration of the experiment. Chambers were octagonal in shape and had alternating stainless steel and Plexiglas walls. The stainless steel walls contained inserts for the food hopper, drinking spout, a house light, two response levers, and two stimulus light panels (Coulbourn Instruments, Lehigh Valley, PA). The house light (4.76 W) was located at the top of the chamber. The food and water inserts were positioned on the two panels opposite the response levers. A stimulus light panel was placed directly above each response lever, and each panel consisted of three colored LED lights (4.76 W), that were simultaneously illuminated upon a lever press. All operant chambers were enclosed in custom-made, sound-attenuating melamine-coated wooden boxes containing a fan that provided ventilation and white noise. Each cocaine infusion system consisted of a syringe pump (model PHM-100, Med Associates, St Albans, VT) with a 30 ml syringe that was connected to a blunted 22-gauge needle tip and attached to Tygon tubing (1.52 mm o.d., 0.51 mm i.d., Fisher Scientific, Springfield, NJ). The free end of the tubing was connected to a swivel (050–0022, Alice King Chatham, Hawthorne, CA) that was mounted at the top center of the operant chamber. The swivel was secured to a spring-covered tether (C313CS, Plastic Products, Roanoke, VA) that extended down into the operant chamber and was attached to the rats covance infusion harness (Instech Laboratories, Plymouth Meeting, PA). IBM-compatible computers equipped with a Med-PC interface (Med Associates, St Albans, VT) were used for programming, data collection, and data storage during all experimental sessions.

Procedure

Surgery. Aseptic techniques were used for all surgical procedures. Rats were anesthetized with ketamine (60 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). Rats were supplemented with doxapram (5 mg/kg, s.c.) to stimulate/stabilize respiration, and atropine (0.4 mg/ml, 0.15 ml, s.c.) was given to prevent bradycardia and to reduce pharyngeal and tracheal secretions (eg, mucus) that are normally removed by a swallowing reflex in a nonanesthetized animal. Once anesthetized, rats received a bilateral OVX and their right jugular vein was catheterized to allow for i.v. cocaine self-administration. For catheterization, an incision was made ventrally at an area rostral to the thorax and lateral to the trachea, and connective tissues were separated with hemostats to allow for isolation of the right jugular vein. The tapered end of a chronic, indwelling silastic catheter that had been previously disinfected in betadine was then inserted into the vein and secured with silk suture. The free end of the catheter was tunneled s.c., exited in the midscapular region, and attached to a cannula (C3236, Plastic One, Roanoke, VA) incorporated in a covance harness that was secured onto the rat. The free end of this cannula was capped until the following day, when it was attached the tether/swivel attachment. For the OVX surgery, a bilateral incision of the dorsal abdomen was made with a scalpel, and muscle wall was separated with hemostats to allow access to the abdominal cavity. Hemostats were used to localize the ovaries, which were gently externalized and separated from the surrounding adipose tissue before being removed with a scalpel. The remaining fallopian tubes were clamped until any bleeding had ceased, the area was rinsed with sterile saline, and tissues were then returned to the abdominal cavity. For both surgeries, previously separated

muscle wall and outer incisions were sutured with chromic gut (Ethicon Inc., Somerville, NJ), and povidine was applied to outer incisions to prevent infection.

Rats were given a total of 10 days to recover from surgery. After recovery from anesthesia, buprenorphine (0.5 mg/kg, s.c., b.i.d.) was given for 2 days as a postoperative analgesic. During this time, rats were also given the antibiotic gentamicin (2 mg/kg, i.v.) and heparinized saline (50 USP U/ml, 0.3 ml/rat). Heparinized saline (0.3 ml/rat/day) was also given for the following week of recovery to reduce blood clotting in the catheter. After recovery, syringe pump tubing was connected to the free (capped) end of the tether/swivel assembly, and experimental sessions commenced. During the experiment, rats were weighed every week, and catheter patency was assessed by injecting (i.v.) a combination of ketamine (100 mg/ml), midazolam (5 mg/ml), and saline (3 : 3 : 14 ratio, 0.10–0.20 ml/rat). If this drug combination produced an immediate loss of the righting reflex upon administration, the catheter was considered to be patent. If not, the experimental session was terminated, the rat was implanted with a new catheter into its left jugular vein, allowed to recover for 3 days, and it was then returned to the experiment.

Training. After recovery from surgery, rats were initially trained to lever-press for cocaine reinforcement. Cocaine self-administration training consisted of a fixed-ratio (FR 1) schedule of reinforcement (0.4 mg/kg/inf, i.v.) and took place in daily, 6-h sessions starting at 0900 h. Training sessions were initiated by the illumination of the house light and the noncontingent administration of two cocaine infusions. Responding on either the active (drug) or the inactive (control) lever resulted in illumination of the corresponding stimulus lights above the lever, but only a response on the active lever resulted in a drug infusion. If rats responded on the active lever $\geqslant$75 times/day for 3 days, and had active:inactive lever-press ratios of at least 2 : 1, their priming infusions were discontinued. If, on the following day, rats continued to respond ($\geqslant$50 responses), they progressed into the maintenance phase of the experiment. If they did not continue to respond, their priming infusions were reinstated, and they were subsequently weaned off priming injections until they met the above stated criteria.

Maintenance. For maintenance, self-administration sessions were limited to 2-h/day (starting at 0900 h), and rats were allowed to self-administer cocaine (0.4 mg/kg/inf, i.v.) for 10 days under a FR 1 schedule. Similar to training, the onset of maintenance sessions were signaled by the house light, and lever pressing during the session was accompanied by stimulus light illumination. However, in contrast to training, no priming infusions were given during maintenance. Rats were assigned to treatment groups following maintenance, and groups were matched for cocaine intake to control for potential differences in cocaine-seeking during reinstatement testing that could be related to prior cocaine intake levels (Sutton *et al*, 2000).

Extinction. After maintenance, saline solutions containing heparin (50 USP U/ml) were substituted in place of the cocaine solutions, and rats were allowed to self-administer saline during 14 daily 2-h sessions (0900–1100 h), under FR 1 conditions. All other stimulus conditions (ie, house light onset, stimulus light illumination) remained the same as during the maintenance phase. Under these abstinence conditions, responding decreases gradually, and it typically reaches minimal levels in 8–10 days (Larson and Carroll, 2005; Larson *et al*, 2005; Perry *et al*, 2006).

Pre-reinstatement. At the end of the extinction phase, saline pumps were disconnected from the swivel/tether assembly, and the house light and stimulus lights were turned off. Thus, lever pressing by the rats was without consequence. This phase was included to ensure that responding during reinstatement testing was specifically in response to priming injections (saline or cocaine) and was not influenced by cues (ie, house light, stimulus light, or interoceptive cues from an i.v. infusion). This was carried out because previous studies have demonstrated that exposure to compound stimuli can increase reinstatement responding compared to exposure to a single stimulus, and this can occur even if responding to one of the stimuli (eg, cue light) has previously been extinguished (See *et al*, 1999; Shelton and Beardsley, 2005).

Reinstatement. Reinstatement testing consisted of 6 daily 2-h sessions that began at 0900 with the noncontingent administration of either a saline (S) or a cocaine (C) priming injection (i.p.). Testing progressed over the 6 days according to the following sequence: S C S C S C. The number of responses on the previously active and inactive levers was counted during each session, but there were no programmed consequences associated with lever pressing. In order to assess dose–response functions, cocaine-mediated reinstatement of active lever responding was assessed after the administration of three different doses of cocaine (5, 10, or 15 mg/kg, i.p.). Cocaine dose order was randomly assigned in each rat, and one of the three cocaine doses was assessed on each cocaine testing day, preceded and separated by a day when an i.p. saline priming injection was given.

Hormone treatment. After completion of the maintenance phase, rats were assigned to one of the following treatment groups: (1) OVX + EB (ERα/β, $n = 10$), (2) OVX + PPT (ERα, $n = 11$), (3) OVX + DPN (ERβ, $n = 10$), or (4) OVX + DMSO (control, $n = 10$). Groups were matched according to their cocaine self-administration during maintenance, so that differences in responding during reinstatement could be directly attributed to treatment and not to differences in drug intake during maintenance. Treatment with DMSO, EB, PPT, or DPN began on the first day of pre-reinstatement; thus, rats were OVX for an average ($\pm$SEM) of 47.07 ($\pm$1.44) days prior treatment. All treatments were administered at 0830 on each day of testing, and treatment lasted a total of 9 days, with the final injection being given on the last day of reinstatement testing. The duration of hormone treatment used in this study was chosen based on a previous study (Larson *et al*, 2005) indicating that 9 days of EB treatment enhanced the magnitude of cocaine-induced reinstatement responding in OVX female rats.

To confirm the appropriate hormonal condition in each rat, vaginal cytology was assessed at two time points in this experiment. During these time points, vaginal samples were taken at 1400 h each day, transferred to slides, stained with methylene blue, and cover slipped. The first set of samples was taken during recovery from surgery, for 5 days before the initiation of cocaine self-administration training. This was carried out in order to verify completeness of the OVX surgery and dissipation of endogenous hormones, as indicated by the predominance of leukocytes and/or the absence of epithelial cells in the samples. Only rats that had slides that verified a complete OVX were used in the study. After obtaining the first set of samples, swabbing was discontinued, and it resumed during hormonal treatment, which occurred during the pre-reinstatement and reinstatement phases of the experiment (9 days total). This second set of samples was taken in order to confirm that the rats' hormonal status was consistent with its treatment group. For example, OVX rats treated with DMSO (control) had cytology similar to untreated OVX rats (predominance of leukocytes), rats treated with EB (ERα/β) had cytology showing a predominance of cornified epithelial cells, rats treated with PPT (ERα) had samples that consisted mainly of nucleated and/or cornified epithelial cells, and rats treated with DPN (ERβ) had slides similar to untreated OVX rats, although the presence of polymorphic epithelial cells was also noted in these animals.

Data analyses. All statistical analyses were conducted using GB Stat (Dynamic Microsystems Inc., Silver Spring, MD). Dependent measures were the number of infusions self-administered during maintenance and extinction, and the number of responses made during reinstatement. The number of cocaine infusions self-administered across the 10-day maintenance phase was analyzed with a two-way repeated measure analysis of variance (ANOVA), with group as the between-subject factor, and maintenance day as the within-subject, repeated factor. The number of saline infusions self-administered across the 14-day extinction phase was also analyzed using a two-way repeated measure ANOVA, with the same between- and within-group factors (ie, group and day). The number of active and inactive lever responses made during reinstatement was analyzed separately using two-way repeated measure ANOVAs, with treatment as the between-group factor and dose as the within-group, repeated factor. When appropriate, *post hoc* tests were conducted using Fisher's LSD protected *t*-tests. Results were considered significant if $p<0.05$.

RESULTS

Training

A total of 62 OVX female rats underwent training for cocaine self-administration. Of these, a total of 41 rats (66%) reached the training criteria, and those that did so reached the criteria in an average ($\pm$SEM) of 13.07 ($\pm$1.44) days. These rats were then allowed to self-administer cocaine across a 10-day maintenance period, and they were separated into treatment groups so that each group had similar cocaine self-administration histories before treatment and reinstatement testing. Retrospective analysis indicated that matching groups based on their cocaine self-administration during maintenance resulted in four groups of OVX rats that did not differ in their time to reach the training criteria for cocaine self-administration (Figure 1). The average ($\pm$SEM) number of days needed to reach the training criteria was 14.8 ($\pm$3.28), 10.5 ($\pm$1.72), 10.73 ($\pm$2.93), and 16.5 ($\pm$3.50) days for OVX + DMSO (control), OVX + EB (ERα/β), OVX + PPT (ERα), and OVX + DPN (ERβ) groups, respectively.

Maintenance and Extinction

As depicted in Figure 2, matching OVX rats according to their self-administration behavior during maintenance resulted in four groups that showed no significant differences in cocaine self-administration as a function of group ($p>0.05$) or day ($p>0.05$). The rats in the OVX

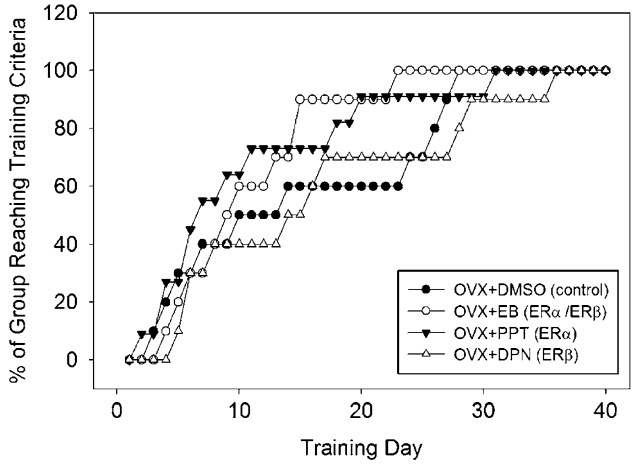

Figure 1 Training. The percent of rats that had reached the training criteria for cocaine self-administration are depicted across training days. The number of days needed to reach the training criteria did not differ between the four groups of OVX rats.

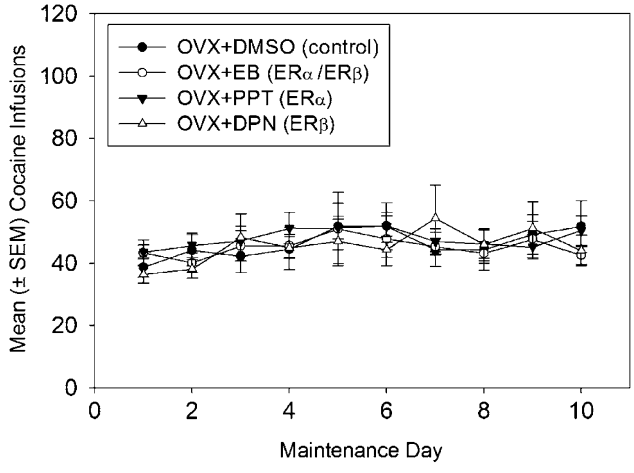

Figure 2 Maintenance. Data points represent the mean ($\pm$SEM) number of cocaine infusions (0.4 mg/kg/inf) self-administered by female OVX rats during 2-h sessions across the 10-day maintenance period. Groups were matched for similar cocaine self-administration in this phase.

+ DMSO (control), OVX + EB (ERα/β), OVX + PPT (ERα), and OVX + DPN (ERβ) groups self-administered an average (±SEM) of 46.3 (±4.7), 45.1 (±3.0), 47.9 (±3.0), and 45.2 (±2.3) infusions per day over the 10-day maintenance period. In all groups, the number of cocaine infusions self-administered from day-to-day remained relatively stable across the maintenance period.

As Figure 3 illustrates, group matching for cocaine intake during maintenance also resulted in four groups of rats that had similar levels of saline self-administration during extinction ($p > 0.05$). There was no difference in how long it took rats to extinguish cocaine-seeking behavior. In all groups of rats, saline replacement led to responding that decreased as a function of extinction day ($F_{13,573} = 26.094$, $p < 0.0001$), and responding reached asymptotic low levels by extinction days 8–9.

Table 1 indicates that the number of responses made on the inactive lever during maintenance and extinction was low relative to those made on the active lever during both maintenance ($F_{1,87} = 188.489$, $p < 0.0001$) and extinction ($F_{1,87} = 188.489$, $p < 0.0001$). *Post hoc* comparisons revealed that this difference was found in all groups (*$p < 0.05$, Table 1). Similar to what was found for the number of infusions self-administered, there were no group differences in the number of responses made on the active lever during maintenance or extinction. There were also no differences in inactive lever responding during this time.

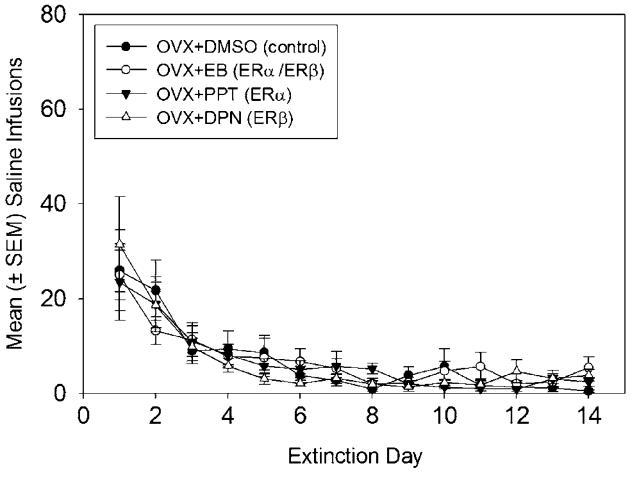

Figure 3 Extinction. Data points represent the mean (±SEM) number of saline infusions self-administered by female OVX rats during 2-h sessions across the 14-day extinction period.

Pre-reinstatement and Reinstatement

During the 3-day pre-reinstatement period, there were no significant within- or between-group differences in the number of responses made on either the active or the inactive levers (Table 1). Responding on both the previously active and inactive levers was low during this time, and it was comparable to what was found at the end of the extinction phase. During reinstatement testing, there were no differences in responding on the previously active (cocaine paired) lever across days where saline priming injections were given; therefore, data from saline testing days were collapsed within groups. Figure 4 illustrates that, in all groups of rats, responding on the previously active lever was low after saline priming (0 mg/kg cocaine), and it was comparable to the number of responses made during the pre-reinstatement phase. In contrast, exposure to cocaine priming injections dose dependently increased the number of times the rats responded on the previously active lever ($F_{3,163} = 21.110$, $p < 0.0001$). The amount of active lever responding after cocaine priming injections also differed as a function of treatment group ($F_{3,163} = 3.158$, $p = 0.036$). *Post hoc* analyses revealed that there were group differences in the lowest dose of cocaine needed to reinstate active lever responding. Both the OVX + EB (ERα/β) and OVX + DPN

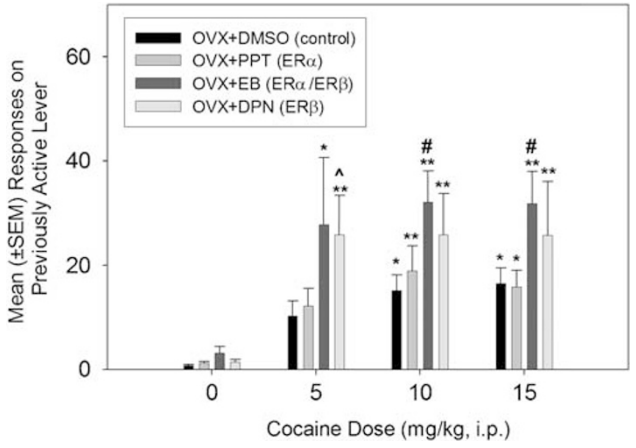

Figure 4 Reinstatement—active lever responding. Data points represent the mean (±SEM) number of responses on the previously active (cocaine paired) lever. *$p < 0.05$, **$p < 0.01$ compared to 0 mg/kg dose within group, #$p < 0.05$ OVX + EB compared to OVX + DMSO and OVX + PPT groups, ^$p < 0.05$ OVX + DPN compared to OVX + DMSO and OVX + PPT groups.

Table 1 Mean (±SEM) Responses on Active and Inactive Levers

	OVX+DMSO		OVX+EB		OVX+PPT		OVX+DPN	
	Active	Inactive	Active	Inactive	Active	Inactive	Active	Inactive
Maintenance	81.55 (±14.9)*	16.75 (±6.7)	66.67 (±5.1)*	8.36 (±2.5)	64.15 (±5.1)*	8.57 (±2.4)	65.36 (±6.1)*	9.30 (±2.7)
Extinction	9.41 (±2.5)*	2.62 (±1.1)	10.21 (±2.2)*	3.80 (±1.2)	8.36 (±2.1)*	3.03 (±0.8)	8.02 (±1.6)*	1.88 (±0.5)
Pre-reinstatement	1.07 (±0.4)	1.22 (±1.1)	2.60 (±0.9)	1.54 (±0.7)	1.85 (±0.6)	1.03 (±0.3)	1.47 (±0.5)	1.38 (±0.4)

*$p < 0.05$ active *vs* inactive lever responding, within group and phase.

(ERβ) groups had significant increases in active lever responding after the 5 mg/kg cocaine prime, whereas at least 10 mg/kg cocaine was needed to produce significant increases in active lever responding in the OVX + DMSO (control) and OVX + PPT (ERα) groups ($^{*}p<0.05$, $^{**}p<0.01$ *vs* 0 mg/kg dose, Figure 4). *Post hoc* comparisons also revealed group differences in the magnitude of active lever responding after cocaine priming injections. The OVX + EB group had more active lever responding after the 10 and 15 mg/kg doses of cocaine when compared to either the OVX + DMSO or OVX + PPT groups ($^{\#}p<0.05$, Figure 4), and OVX + DPN group had higher active lever responding compared to the OVX + DMSO and OVX + PPT groups after 5 mg/kg cocaine ($^{\wedge}p<0.05$, Figure 4).

Figure 5 depicts the number of responses made on the previously inactive (not cocaine-paired) lever during reinstatement testing after rats were exposure to the saline or cocaine priming injections. Analysis revealed that, similar to active lever responding, responding on the inactive lever increased as a function of cocaine dose ($F_{3,163}=9.068$, $p<0.0001$). *Post hoc* analyses indicated that inactive lever responding was increased after priming with the 5, 10, and 15 mg/kg doses of cocaine in OVX + EB (ERα/β) rats, and in response to the 15 mg/kg cocaine priming dose in OVX + PPT (ERα) rats ($^{*}p<0.05$, $^{**}p<0.01$ *vs* 0 mg/kg dose, Figure 5). The amount of inactive lever responding after cocaine also differed between groups ($F_{1,163}=3.823$, $p=0.0176$). The cocaine-induced increase in inactive lever responding after the 15 mg/kg cocaine prime was higher in the OVX + EB group compared to both OVX + DMSO (control) and OVX + DPN (ERβ) groups ($^{\#}p<0.05$, Figure 5). Similarly, the OVX + PPT group made more inactive lever responses after the 15 mg/kg cocaine prime than either the OVX + DMSO and OVX + DPN groups ($^{\dagger}p<0.05$, Figure 5).

Given that cocaine led to increases in both active and inactive lever responding in OVX + EB (ERα/β) rats, an additional analysis was conducted to determine whether there were differences in the magnitude of cocaine-induced responding on these levers in the OVX + EB group. A two-way repeated measures ANOVA confirmed that lever responding increased as a function of cocaine priming dose ($F_{3,79}=9.463$, $p<0.0001$), but it also indicated that cocaine priming produced greater increases in active lever responding than inactive lever responding ($F_{1,79}=20.642$, $p<0.0001$). *Post hoc* analysis confirmed that active lever responding was significantly greater than inactive lever responding after priming with the 10 and 15 mg/kg cocaine doses ($p<0.05$). This indicates that the enhanced active lever responding in the OVX + EB group compared to OVX + DMSO (control) and OVX + PPT (ERα) groups at these doses was specific for cocaine-seeking behavior, and it was not a result of generalized activity increases in the OVX + EB group.

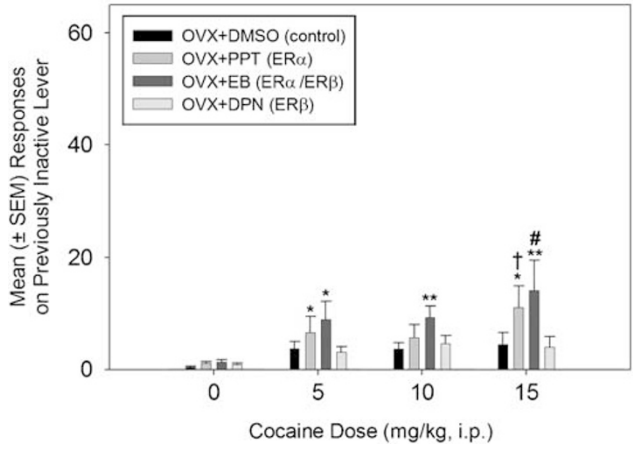

Figure 5 Reinstatement—inactive lever responding. Data points represent the mean (±SEM) number of responses on the previously inactive lever. $^{*}p<0.05$, $^{**}p<0.01$ compared to 0 mg/kg dose within group, $^{\#}p<0.05$ OVX + EB compared to OVX + DMSO and OVX + DPN groups, $^{\dagger}p<0.05$ OVX + PPT compared to OVX + DMSO and OVX + DPN groups.

DISCUSSION

ER Activation and Active Lever Responding during Reinstatement

In the present study, we examined the role of ERα and ERβ in estrogen's ability to enhance cocaine-induced reinstatement of cocaine-seeking behavior in female OVX rats. Consistent with previous work (Larson *et al*, 2005), we found that administration of EB, a mixed ERα/ERβ agonist, increased the magnitude of responding on the previously active (cocaine paired) lever compared to OVX + DMSO controls. We have expanded on this previous work to show that OVX rats treated with EB also reinstate cocaine-seeking behavior after lower priming doses of cocaine. That is, OVX + EB rats reinstated responding after a priming injection of 5 mg/kg cocaine, whereas a dose of 10 mg/kg cocaine was needed to produce significant reinstatement in the OVX + DMSO group. These findings indicate that estrogen can effectively promote the reinstatement of extinguished cocaine-seeking behavior in OVX female rats, even when cocaine self-administration has not been in effect for an extended period of time.

Similar to OVX + EB (ERα/β) rats, OVX rats treated with a 1.0 mg/kg dose of the ERβ-selective agonist DPN also reinstated cocaine seeking after lower priming doses of cocaine, and they showed more cocaine-seeking behavior during reinstatement compared to the OVX + DMSO controls. The magnitude of responding during reinstatement was also comparable between the OVX + EB and OVX + DPN groups. In contrast, rats treated with a 1.0 mg/kg dose of the ERα-selective agonist PPT showed reinstatement of active lever responding that resembled that of the OVX + DMSO controls, indicating that the ERα activation produced by this dose of PPT did not influence the amount of cocaine-seeking behavior. The dose of PPT and DPN that we used in this study (1.0 mg/kg) is biologically effective (Harris *et al*, 2002; Frasor *et al*, 2003; Lund *et al*, 2005) and selectively activates the ERα and ERβ subtypes, respectively (Lund *et al*, 2005). Furthermore, at the 1.0 mg/kg dose, both compounds can reach the brain within 30 min of peripheral administration (Harris *et al*, 2002; Lund *et al*, 2005), which corresponds to the timing of the treatment regimen used in this study. Overall, the results obtained during reinstatement testing strongly suggest that estrogen-mediated activation of ERβ, but not ERα,

promotes the reinstatement of cocaine-seeking behavior in OVX female rats. Interestingly, it has been shown that tamoxifen, a partial agonist/antagonist at ERα, but pure antagonist at ERβ (Barkhem *et al*, 1998), effectively reduced acquisition of cocaine self-administration in intact female rats (Lynch *et al*, 2001). Thus, activation of ERβ may promote cocaine seeking across several phases of drug abuse, and ERβ may be the basis for sex differences in drug-taking and drug-seeking behavior.

ER Activation and Inactive Lever Responding during Reinstatement

In the present study, although cocaine priming injections led to significantly more responding on the active compared to the inactive lever, cocaine priming injections were not without effect on inactive lever responding. Specifically, cocaine dose dependently increased inactive lever responding, and these increases were found to be significant in both the OVX + EB (ERα/β) and OVX + PPT (ERα) groups, but not in the OVX + DPN (ERβ) or OVX + DMSO (control) groups. In self-administration paradigms, inactive lever responding is often considered to be a measure of general locomotor activity levels. Thus, in addition to promoting reinstatement of cocaine-seeking behavior, cocaine may have also caused increased locomotor activity in these animals. The finding that the OVX + EB rats had greater inactive lever responding than OVX + DMSO rats is consistent with several studies that found that the locomotor activity produced by cocaine, at similar bolus doses that were used in the present study, was enhanced by estrogen treatment (eg, Sell *et al*, 2000, 2002; Perrotti *et al*, 2001; Chin *et al*, 2002). Interestingly, in contrast to what was found for active lever responding, the effect of EB on inactive lever responding was also found in rats pretreated with PPT, but not those pretreated with DPN. These findings indicate that ERα and ERβ have differential roles in mediating estrogen's influence on locomotor activity and cocaine-seeking behavior, with ERα exerting greater influence on locomotor activity and ERβ exerting greater influence on cocaine-seeking behavior. It also suggests that estrogen's ability to enhance these effects may result from different underlying mechanisms and/or pathways in the brain.

Possible Interpretations of the Results

The relative differences found in active lever responding after cocaine priming injections suggest that ERβ is important for promoting the vulnerability to reinstate cocaine-seeking behavior. One way that activation of ERβ by either EB or DPN may have facilitated the reinstatement of cocaine-seeking behavior was by enhancing the rewarding effects or salience of the cocaine priming stimuli, resulting in a greater impact on subsequent cocaine-seeking behavior. As the magnitude of active lever responding during reinstatement has been used as a measure of an animal's motivation to obtain cocaine (De Wit and Stewart, 1981; Self *et al*, 1996), another possibility is that activation of ERβ by EB or DPN served to enhance the incentive-motivational state of the rat. Some support for this idea comes from one study that used a progressive ratio schedule of reinforcement to assess motivation and found that under certain conditions EB could enhance responding for cocaine under this schedule (Lynch and Taylor, 2005). However, more research is needed to specifically examine the effects of estrogen and the role of ERα and ERβ on cocaine-associated motivational states.

As previously mentioned, one interpretation of inactive lever responding is that it reflects general locomotor activity levels. An alternative interpretation could be that inactive lever pressing reflected generalized or nonspecific responding. This nonspecific responding may indicate an error in the rat's ability to consistently recall which lever had previously been associated with cocaine, particularly when such correct recall did not result in cocaine reinforcement. In all groups, active lever pressing during reinstatement was greater than inactive lever pressing, suggesting that all groups of rats had retained the previously learned association between active lever pressing and delivery of the cocaine reward. However, it may be that activation of ERβ with DPN resulted in a more consistent retention of this previously learned association than was produced by activation of ERα by PPT. Studies that have examined the role of ERs in estrogen's effects on learning and memory have suggested that the ERβ subtype is important for learning and memory, including spatial learning (Rissman *et al*, 2002) and retention of spatial and inhibitory avoidance memory tasks (Rhodes and Frye, 2006). Conversely, activation of the ERα subtype may inhibit learning (Fugger *et al*, 2000). Therefore, it is possible that differences in active and inactive lever responding between the OVX + PPT and OVX + DPN groups were due to differences in memory recall between the two groups. It may be that both groups were similarly motivated to seek cocaine, but the DPN-treated rats had a better ability than the PPT-treated rats to consistently recall which lever had previously been associated with the cocaine reward. However, if differences in ERα and ERβ activation led to differences in memory recall, we would have expected to find that treatment with PPT increased inactive lever responding after priming with both the 10 and 15 mg/kg doses of cocaine. It also might be expected that rats treated with EB (ERα/β) would have shown active and inactive lever responding at a level that was in-between that of OVX + PPT and OVX + DPN rats. In both respects, this was not the case. Unfortunately, the interpretations of the results are further complicated by the possibility of an interaction of ERα and ERβ effects on reward, motivation, and/or memory recall.

Possible Brain Regions Involved in Estrogen Effects on Reinstatement

Both ERα and ERβ are expressed in brain regions (eg, VTA, amygdala, and prefrontal cortex) that have been implicated in the reinstatement of drug-seeking induced by various stimuli, including stress, drugs, and cocaine-associated cues (See, 2002; Shalev *et al*, 2002; Shaham *et al*, 2003; Aston-Jones and Harris, 2004; Bossert *et al*, 2005; Weiss, 2005). Although the pathways underlying relapse induced by these stimuli differ, there is some overlap in brain regions such as the prefrontal cortex and nucleus accumbens (Self and Nestler, 1998; Kalivas and McFarland, 2003; Kalivas and Volkow, 2005). Given the results of the present study, it is

unlikely that the effects of estrogen on reinstatement are a result of ERβ activation in the amygdala, as this region has been implicated in reinstatement of drug-seeking behavior induced by cocaine-associated cues, but not for reinstatement elicited by priming injections of cocaine (McFarland and Kalivas, 2001; Kalivas and McFarland, 2003; Bossert *et al*, 2005). This idea is supported by recent studies in rats indicating that gonadal hormones (eg, estrogen) influence reinstatement induced by cocaine (Kippin *et al*, 2005; Larson *et al*, 2005), but not reinstatement elicited by exposure to cocaine-associated cues (Fuchs *et al*, 2005). Given that cocaine-induced reinstatement involves more direct activation of the mesolimbic pathway (ie, VTA to nucleus accumbens), it is likely that estrogen may be acting on receptors in these areas.

Both ERβ mRNA and protein have been consistently found in the VTA (Shughrue *et al*, 1997; Shughrue and Merchenthaler, 2001; Creutz and Kritzer, 2002), and these receptors have been localized both on A10 dopamine neurons projecting to the striatum, as well as on nondopaminergic neurons within the VTA (Creutz and Kritzer, 2002). This, along with the apparent lack of ERα expression in this region, suggests that estrogen may promote the reinstatement of cocaine-seeking behavior by acting on ERβ in the VTA. Estrogen activity at ERβ in the VTA could potentially increase dopamine function by stimulating the activity of dopamine neurons, or conversely by reducing inhibitory influences on dopamine neurons. For example, activation of $GABA_B$ receptors in the VTA decreases cocaine self-administration (Brebner *et al*, 2000), and estrogen treatment reduces $GABA_B$-mediated G-protein activation in the VTA (Febo and Segarra, 2004). Similarly, activation of ERβ in the nucleus accumbens may also be involved in estrogen's effects on reinstatement. It has been suggested that D_2-like dopamine receptors in the nucleus accumbens may be crucial for the reinstatement of drug-seeking behavior (Self and Nestler, 1998), and ERβ, but not ERα, mediates estrogen-induced increases in D_2 receptors in this region (Le Saux *et al*, 2006). Another possible site where estrogen may be acting to promote cocaine-induced reinstatement is the prefrontal cortex. The prefrontal cortex has been implicated in stress, cue, and drug-induced reinstatement (Bossert *et al*, 2005). Although no studies to date have specifically looked at ERα and ERβ localization in this specific region of the cortex, there is evidence that ERβ may be the predominant ER subtype in various cortical regions (Shughrue *et al*, 1997; Laflamme *et al*, 1998). Thus, although more work is needed to understand the how estrogen activation of ERβ may lead to increased cocaine-seeking behavior, it is likely that these effects involve activation of ERβ in regions of the brain associated with cocaine reward.

Consideration of Study Limitations

Although the results of the present study mainly indicate that ERβ mediates estrogen's effects on the reinstatement of cocaine-seeking behavior, whereas ERα may be more involved in the locomotor-activating effects of cocaine, there are a few limitations of the present study that should also be considered. First, only one dose (1.0 mg/kg) of the ERα- and ERβ-selective agonists were examined in this study. It is unknown whether consistent results would have been found if higher or lower doses of the selective agonists were tested. For example, it is possible that higher doses of PPT (ERα) would have also facilitated the reinstatement of cocaine-seeking behavior. It is also possible that the increase in cocaine-seeking behavior seen with DPN (ERβ) treatment was specific to the 1.0 mg/kg dose. Additionally, it is unclear whether coadministration of a 1.0 mg/kg dose of both PPT and DPN would have actually produced comparable effects to that found for rats treated with EB (ERα/β).

Another limitation of the present study was that the ERα-selective agonist PPT had a far greater selectivity (300 : 1 ERα : ERβ) than the ERβ-selective agonist DPN (70 : 1 ERβ : ERα). Therefore, while it is likely that the effects of PPT were selectively owing to its action at ERα, it is possible that the reported effects of DPN were not solely owing to ERβ activation. For example, it has been shown that DPN can only stimulate ERβ to about 45% of the activity of estradiol, after which it may also stimulate ERα (Meyers *et al*, 2001). Thus, it may be that the dose of DPN that we used in the present study may have produced activation of both ERα and ERβ subtypes. However, if treatment with DPN did activate a significant amount of ERα, we would have expected to see an associated change in the vaginal cytology (ie, appearance of cornified epithelium), which we did not. Also, it has been reported by another group that daily administration of a 1.0 mg/kg dose of PPT increased uterine weight and upregulated progestin receptor immunoreactivity in the brain, whereas the same dose of DPN did not (Lund *et al*, 2005). Together, these findings suggest that treatment with PPT and DPN selectively activated the ERα and ERβ subtypes, respectively. Even so, as our treatments were longer (9 days) than those used in previously mentioned study (4 days), the possibility that DPN activated both ER subtypes should be considered when interpreting the results of the present study.

A final limitation of the present study was the presence of soy- and alfalfa-derived phytoestrogens, specifically genestein, in the rat food. Although genestein has a greater affinity for the ERβ than the ERα subtype (Kuiper *et al*, 1997; Kostelac *et al*, 2003; Mueller *et al*, 2004; Zhao and Brinton, 2005; McCarty, 2006), it does have the potential to act as a full agonist at both these ER subtypes (Mueller *et al*, 2004; McCarty, 2006). However, as the vaginal cytology of the OVX + DMSO control rats did not show any evidence of vaginal cornification, we do not believe that exposure to phytoestrogens in the rat food led to any significant activation of the ERα subtype. Nevertheless, we cannot exclude the possibility that phytoestrogen exposure in our rats led to some sort of activation of the ERβ subtype. Phytoestrogen exposure may have also led to alterations in the number and/or affinity of ERα or ERβ in the brain. For example, genestein exposure can increase the rate at which bound ERα and ERβ subsequently bind to the estrogen response element, an effect that is slightly more pronounced for ERβ than for ERα (Kostelac *et al*, 2003). Thus, it is possible that increases in ERβ activity relative to ERα may have promoted more cocaine-seeking behavior in the OVX + EB (ERα/ERβ) and OVX + DPN (ERβ) groups than would have been found if rats had been fed with a phytoestrogen-free diet. However, if increased activity of ERβ resulting from genestein exposure did indeed underlie the increased

cocaine-seeking behavior, it would be consistent with our conclusion that ERβ plays an important role in estrogen's effect on the reinstatement of cocaine-seeking behavior.

Summary and Conclusions

In summary, the present study confirmed previous findings (Larson *et al*, 2005) indicating that EB (ERα/β) treatment increases the ability of cocaine priming to elicit reinstatement of cocaine-seeking behavior in OVX female rats. Activation of ERβ by the selective agonist DPN produced an enhancement of cocaine-induced reinstatement of active lever responding that was similar to EB, whereas activation of ERα by the selective agonist PPT had no effect on cocaine-seeking behavior relative to controls. In contrast, activation of ERα by PPT led to increases in inactive lever responding, suggesting that ERα may be involved in EB-related increases in locomotor activity after cocaine. Together, these findings indicate that activation of ERβ, but not ERα, plays in important role in estrogen's ability to promote the reinstatement of cocaine-seeking behavior. Whether this effect is due to alterations in reward, motivation, memory recall, or an interaction of these processes warrants further investigation. Overall, the results suggest that activation of ERβ may be important for the expression of sex differences in the vulnerability to relapse to cocaine.

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