Neuropsychopharmacology (2007) 32, 522–530
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www.neuropsychopharmacology.org

Estradiol-Induced Conditioned Place Preference may Require Actions at Estrogen Receptors in the Nucleus Accumbens

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Intrinsic rewarding effects of estradiol (E_2) may underlie some of the sex differences that emerge postpuberty for the prevalence of drug use and behavioral responses to drugs, but the effects and mechanisms of E_2 for reward have not been well characterized. Conditioned place preference (CPP), as measured by the time spent on the nonpreferred/drug-associated side of the chamber, was utilized as a functional assay to investigate the effects and mechanisms of E_2 in the nucleus accumbens for reward. To determine whether intracellular estrogen receptors (ERs) are important for E_2-induced CPP, rats were administered E_2 (10 μg; subcutaneously (s.c.)), which produced CPP in each experiment, and/or ER blockers, such as tamoxifen (Experiment 1), ICI 182,780 (Experiment 2), or antisense oligonucleotides targeted to ERs (Experiment 3). Experiment 1: E_2 significantly increased the time spent on the originally nonpreferred side of the chamber. Coadministration of tamoxifen (10 mg/kg; s.c.) attenuated effects of E_2 to produce a CPP, but tamoxifen alone, increased time spent on the nonpreferred side. Experiment 2: coadministration of ICI 182,780 (10 μg/μl) to the nucleus accumbens attenuated effects of E_2 to enhance CPP and did not produce a CPP when administered alone. Experiment 3: coadministration of s.c. E_2 with ER antisense oligonucleotides to the nucleus accumbens significantly decreased time spent on the nonpreferred side and expression of ERs in the nucleus accumbens compared to scrambled antisense oligonucleotides or saline vehicle administration. Thus, E_2's rewarding effects may involve actions at ERs in the nucleus accumbens.
Neuropsychopharmacology (2007) **32,** 522–530. doi:10.1038/sj.npp.1301124; published online 7 June 2006

Keywords: estrogen; tamoxifen; reward; affect; anxiety; SERMs

INTRODUCTION

There are sex differences in vulnerability to drug abuse among people and in animal models (SAMHSA, 2004; reviewed by Carroll *et al*, 2004). Clinical and animal model studies suggest that adult females may also be more vulnerable to drugs of abuse than are their male counterparts. Women begin cocaine use at a younger age than do men (Griffin *et al*, 1989) and can become addicted to abused substances, such as cocaine, opiates, and alcohol, faster than do men (Lex, 1991). Similarly, female rodents demonstrate quicker acquisition of cocaine, methamphetamine, and nicotine intravenous self-administration than do males (Caggiula *et al*, 2002; Carroll *et al*, 2001; Roth and Carroll, 2004). Thus, these data suggest that females may be more vulnerable to effects of drugs of abuse. However, potential mechanisms for these effects are yet to be determined.

One possible reason for sex differences in drug abuse may involve estradiol's (E_2) effects to underlie or influence responses to drugs of abuse among people and animals. There are differences across the menstrual cycle in women's response to drugs. For instance, subjective responses to amphetamine (ie feelings of euphoria, liking/wanting, energy, and intellectual ability) are enhanced during the late follicular phase, when E_2 levels are rising, compared to the early follicular or luteal phase, when E_2 levels are lower (Justice and de Wit, 1999, 2000a). Indeed, there is a positive correlation between subjective responses to amphetamine and E_2 levels (Justice and de Wit, 1999). Furthermore, transdermal administration of E_2 to women in the follicular phase enhances subjective responses to amphetamine (Justice and de Wit, 2000b). Additionally, rodent models support a role for E_2 in drug abuse. Naturally receptive rodents that have moderate physiological levels of E_2 have increased hyperactivity after cocaine administration than do diestrous rats with lower levels of E_2 (Sell *et al*,

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Received 6 February 2006; revised 15 March 2006; accepted 16 March 2006
Online publication: 12 May 2006 at http://www.acnp.org/citations/Npp051206060072/default.pdf

2002). Removal of the primary source of E_2, the ovaries (OVX), attenuates amphetamine-induced hyperactivity, cocaine-induced behavioral sensitization, and intravenous cocaine self-administration, which can be reversed by E_2 replacement (Becker, 1990; Lynch *et al*, 2001; Peris *et al*, 1991). Thus, E_2 may influence responses to drugs of abuse.

Drug abuse and neuropsychiatric disorders are often comorbid and, notably, E_2 influences affect among women and in animal models (Walf and Frye, 2006). Women who abuse alcohol, cocaine, opiates, and/or nicotine have higher rates of mood and/or anxiety disorders than do men (Brooner *et al*, 1997; Borrelli *et al*, 1999; Cornelius *et al*, 1995; Lundy *et al*, 1995; McCance-Katz *et al*, 1999). Among women who have anxiety/mood disorders, E_2 therapy can enhance mood, but is dependent on the regimen utilized (Ahokas *et al*, 2001; Gregoire *et al*, 1996; Saletu *et al*, 1995; Schleifer *et al*, 2002; Smith *et al*, 1995). A similar pattern is observed in animal models. In naturally receptive rats, or OVX rats administered E_2 regimen that produce physiological E_2 levels, anxiety and depression behaviors are decreased (Estrada-Camarena *et al*, 2003; Frye *et al*, 2000; Frye and Walf, 2002, 2004; Frye and Wawrzycki, 2003; Nomikos and Spyraki, 1988; Rachman *et al*, 1998; Slater and Blizard, 1976; Walf and Frye, 2005a, b, 2006). Thus, these data suggest that E_2 can alter affective responses.

E_2 may have rewarding effects in the conditioned place preference (CPP) paradigm, which is used to elucidate effects of substances on reward (White and Carr, 1985). Subcutaneously (s.c.) administered E_2 regimen that result in physiological E_2 levels in the nucleus accumbens of OVX rats produces a CPP (Frye and Rhodes, 2006). The nucleus accumbens is critical for CPP and is a target of E_2. For example, E_2 increases c-jun-immunoreactivity in the nucleus accumbens (Zhou and Dorsa, 1994). Whether E_2 has requisite actions in the nucleus accumbens to mediate CPP is not known.

Given that E_2 can alter responses to drugs of abuse, as well as produce rewarding effects, it is important to determine the mechanisms for these effects. E_2 alters other reward-related processes that increase dopamine release in the nucleus accumbens or striatum of female rats, such as wheel-running, locomotion, and pacing of copulatory contacts (Becker *et al*, 1987, 2001; as reviewed by Becker, 1999; Jenkins and Becker, 2003; Mermelstein and Becker, 1995; as reviewed by Lynch *et al*, 2002; Pfaus *et al*, 1995; Xiao and Becker, 1997). Pharmacological blockade of estrogen receptors (ERs) in these regions with the selective antagonist, ICI 182,780, but not tamoxifen (which can have both agonistic and antagonistic effects at ERs) attenuates E_2-facilitated pacing behavior (Xiao and Becker, 1997; Xiao *et al*, 2003). Thus, one mechanism by which E_2 may influence reward processes is through actions at ERs.

A specific binding site for E_2 was identified over 40 years ago (Jensen and Jacobsen, 1962) and eventually became known as ERs. More recently, another E_2 binding site with which E_2 interacts to have its functional effects has been identified (as reviewed by Kuiper *et al*, 1998). The original binding site is now referred to as ERα and the newly identified binding site is referred to as ERβ. The nucleus accumbens expresses ERs (referring to both ERα and ERβ; Shughrue *et al*, 1997, 1998). Thus, we utilized a number of pharmacological tools to block ER action in the nucleus accumbens to address E_2's mechanisms in this region for reward.

In the present study, we investigated whether E_2-induced CPP requires actions at ERs in the nucleus accumbens. Rats were s.c. administered E_2 and/or ER blockers, such as tamoxifen s.c. (Experiment 1), ICI 182,780 to the nucleus accumbens (Experiment 2), or antisense oligonucleotides targeted toward the originally identified binding site of E_2 infused to the nucleus accumbens (Experiment 3). We hypothesized that if actions at ERs in the nucleus accumbens are required for E_2's rewarding effects, then coadministration of E_2 in conjunction with ER blockers would attenuate E_2-induced CPP.

MATERIALS AND METHODS

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

Animals and Housing

Female Long–Evans rats ($N = 140$), approximately 55 days old, were obtained from the breeding colony in the Social Sciences Building at SUNY-Albany (original stock from Taconic Farms, Germantown, NY). Rats were group-housed (4–5 per cage) in polycarbonate cages ($45 \times 24 \times 21$ cm) in a temperature-controlled room ($21 \pm 1^{\circ}$C) in the laboratory animal care facility. Rats were maintained on a 12/12 h reversed light cycle (lights off 0800 hours) with continuous access to Purina rat chow and tap water.

Surgery

All rats were OVX under Rompun (12 mg/kg; Bayer Corp., Shawnee Mission, KS) and Ketaset (80 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA) anesthesia. For Experiments 2 and 3, rats were stereotaxically implanted with cannulae (23 G) aimed at the nucleus accumbens (from bregma AP = +1.7, ML = $\pm$1.5, DV = −6.0; as per Paxinos and Watson, 1986; Frye *et al*, 2002). All rats were surgerized at least 1 week before testing.

ER Blockade

S.c. tamoxifen. In Experiment 1, rats were coadministered s.c. tamoxifen (10 mg/kg s.c.; Sigma Chemical Co., St Louis, MO), or vehicle (sesame oil and 10% ethanol), in conjunction with sesame oil vehicle or E_2 (10 μg/0.2 cm^3; Sigma). As such, there were four groups ($n = 18$/group) in this experiment: s.c. vehicle + s.c. vehicle, s.c. tamoxifen + s.c. vehicle, s.c. vehicle + s.c. E_2, and s.c. tamoxifen + s.c. E_2. S.c. administration of tamoxifen was utilized because it is an effective, albeit nonselective, ER antagonist that is blood–brain barrier soluble. This tamoxifen regimen has been utilized to block E_2's effects on lordosis (Etgen and Shamamian, 1986) and anxiety behavior (Walf and Frye, 2005b).

Intranucleus accumbens ICI 182,780. In Experiment 2, rats were coadministered ICI 182,780 (10 μg/μl; Tocris

Bioscience, Ellisville, MO), or vehicle (β-cyclodextran) to the shell of the nucleus accumbens, in conjunction with s.c. injections of sesame oil vehicle or E_2 (10 μg/0.2 cm^3). As such, there were four groups in this experiment: intranucleus accumbens vehicle + s.c. vehicle ($n = 14$), intranucleus accumbens ICI 182,780 + s.c. vehicle ($n = 12$), intranucleus accumbens vehicle + s.c. E_2 ($n = 17$), and intranucleus accumbens ICI 182,780 + s.c. E_2 ($n = 10$). ICI 182,780 was utilized because it is a specific ER antagonist and has been used successfully in our laboratory to investigate E_2's effects on mnemonic processes and affective behavior (Frye and Rhodes, 2002; Walf and Frye, 2006).

Intranucleus accumbens antisense oligonucleotides. In Experiment 3, rats were administered saline vehicle ($n = 4$) or full phosphorothioate HPLC-purified mRNA antisense oligonucleotides (Genomechanix, Gainesville, FL). One group of rats was administered ER antisense oligonucleotides ($n = 6$), with the sequence, 5′-CAT-GGT-CAT-GGT-CAG-3′, that spans the putative translation start codon for rat ERα, and knocks down the translation of ERα. Another group of rats was administered scrambled control antisense oligonucleotides ($n = 4$), with a 15-mer sequence (5′-ATC-GTG-GAT-CGT-GAC-3′) that has the same base pairs, but the order randomized, and little or no homology to any mRNA sequences posted at GeneBank (NCBI BLAST search). Rats were infused with ERα antisense oligonucleotides (2 μg/1 μl; McCarthy *et al*, 1993), scrambled control oligonucleotides (2 μg/1 μl), or saline vehicle immediately before administration of E_2, 30 min before placement on the nonpreferred side of the CPP chamber on days 4–5 and 8–9 (as described below in detail).

CPP Paradigm

The CPP paradigm used in the present study was as described previously (Frye and Rhodes, 2006). This paradigm includes the following four phases: habituation, baseline preference test, place preference conditioning, and place preference test.

Habituation (days 1 and 2): Rats were allowed to explore both sides of the conditioning chamber for 30 min each day.

Baseline preference test (day 3): Rats were placed in the chamber for 30 min and the amount of time spent on each side of the chamber was electronically recorded.

Place preference conditioning (days 4–11): A typical place preference conditioning procedure was utilized. Rats were administered drugs with putative conditioning properties (ie E_2 and/or ER blockers in the present experiment) before placement in the nonpreferred side of the chamber (as determined on day 3) on days 4–5 and 8–9. On days 6–7 and 10–11, all rats, irrespective of their experimental condition on conditioning days, were administered vehicle (appropriate to the experiment) immediately prior to placement in the originally preferred side of the apparatus.

Place preference test (day 12): Rats were placed in the apparatus with free access to both sides. The amount of time spent on each side was electronically recorded for a total duration of 30 min. Time on the nonpreferred side is considered an index of CPP.

Site Analyses

After testing in Experiment 2, rats were perfused with 0.9% saline followed by 10% formalin. Brains were removed from the skull, fixed in formalin, and then sliced on a cryostat at 40 μm. Slices were then stained with cresyl violet and infusion location was determined with light microscopy. All but three rats received implants to the shell of the nucleus accumbens, which has previously been described as important for the modulation of CPP by steroids (Frye *et al*, 2002). The three rats that received bilateral implants to the core of the nucleus accumbens were not included in statistical analyses.

Tissue Collection, Dissection, and Digestion

In Experiment 3, rats were readministered their s.c. E_2 and intranucleus accumbens antisense oligonucleotide regimen. Rats were rapidly decapitated, and brains were quickly removed from the skull, and placed on dry ice. Whole brains were stored at −80°C until the nucleus accumbens and pituitary (as a positive control) were dissected out. Cannulae placement was verified during dissection. For dissections, brains were first gently thawed on ice. The nucleus accumbens and pituitaries were each placed in eppendorf tubes containing protease inhibitor cocktail dissolved in distilled water (Roche, 11836145001). Samples were transferred to test tubes on ice and were homogenized. Homogenized samples were then stored in the original eppendorf tubes containing the protease inhibitor overnight at 4°C.

Western Blot Analyses

To verify effective knockdown of ERs with the antisense oligonucleotide strategy utilized, differences in ER protein expression in the nucleus accumbens with Western blotting were assessed. Standard Western blotting techniques were employed using polyclonal anti-rabbit antibodies against ERα (C1355; Upstate Biotechnology, Lake Placid, NY; Tanabe *et al*, 2004; Tanaka *et al*, 2004) to establish the extent to which the antisense oligonucleotides bound to the translation start sites of ERα and inhibited its translation into protein (Schreihofer *et al*, 2002). Western blotting analyses using a monoclonal anti-mouse β-actin antibody (A1978; Sigma) were also performed as a loading control. Briefly, homogenized samples were centrifuged for 20 min at 11 × 1000*g*. Supernatant was separated from the pellet and stored at −20°C. A Bradford assay was then performed to determine protein concentration in each sample (Bradford, 1976). Using this information, 10 μg of protein and sample buffer (50 mM Tris-Cl (pH 6.8), 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol), yielding a final volume of 10 μl, were loaded onto 7.5 × 10.0 cm gels. Samples were resolved on 10% SDS polyacrylamide gel utilizing 25 mM Tris-glycine buffer, pH 8.3, containing 20% methanol at 100 V for 1.5–2 h at room temperature, and electrophoretically transferred to nitrocellulose membrane (Pierce; with pore size of 0.45 μm) using a 25 mM Tris-glycine buffer, pH 8.3, containing 20% methanol, at 100 V for 1.5 h at 4°C. After transfer was complete, nitrocellulose membranes were blocked in

Pierce's blocking buffer (Pierce, Rockford, IL) with 0.05% Tween-20 overnight at 4°C. The membranes were washed in buffer (phosphate-buffered saline with 0.05% Tween-20) on a rocking platform before incubation with the primary antibodies for ERα (1 : 1000 dilution) or β-actin (1 : 1000 dilution) in blocking buffer with 0.05% Tween-20 overnight at 4°C. After incubation with the primary antibody, membranes were washed in buffer on a rocking platform and were incubated with the appropriate horseradish peroxidase-labeled goat anti-rabbit (1 : 3000 dilution for ERα) or goat anti-mouse secondary antibody (1 : 5000 dilution for β-actin) supplied in the chemiluminescence kit (Pierce, Supersignal West FEMTO Max Sensitivity kit; 34095), in blocking buffer with 0.05% Tween-20, for 1 h with agitation. Membranes were then washed in buffer, soaked in the chemiluminescence medium for 5 min, and exposed to X-ray film, and film was developed.

Statistical Analyses

To determine mechanisms and effects of E_2 for producing a place preference in these experiments, three-way analyses of variance (ANOVAs) were utilized in Experiments 1 and 2 to analyze effects of E_2 condition, ER blocker condition, and test occasion (baseline *vs* test). Two-way ANOVAs were used to determine effects of infusion (antisense or scrambled oligonucleotides or vehicle) to OVX, E_2-primed rats and time on CPP. Previous studies in our lab utilizing CPP have suggested that an α level of $P \leqslant 0.05$ is appropriate (Frye and Rhodes, 2006; Frye *et al*, 2002). Where appropriate, ANOVAs were followed by Fisher's *post hoc* tests with Bonferroni corrections to determine group differences. Although sample sizes varied across experiments, *post hoc* power analyses indicated that there was sufficient power in each experiment, minimally 70%.

RESULTS

Experiment 1: Effects of Systemic Tamoxifen (Figure 1)

There were main effects of E_2 condition ($F(1,136) = 5.9$, $P < 0.02$) and test occasion ($F(1,136) = 35.6$, $P < 0.01$) that was owing to E_2 significantly increasing time on the nonpreferred side during testing compared to vehicle. Although there was no significant main effect of tamoxifen, there was an interaction between E_2 and tamoxifen [$F(1,136) = 12.8$, $P < 0.01$] that was owing to tamoxifen attenuating CPP in rats coadministered E_2, but not vehicle.

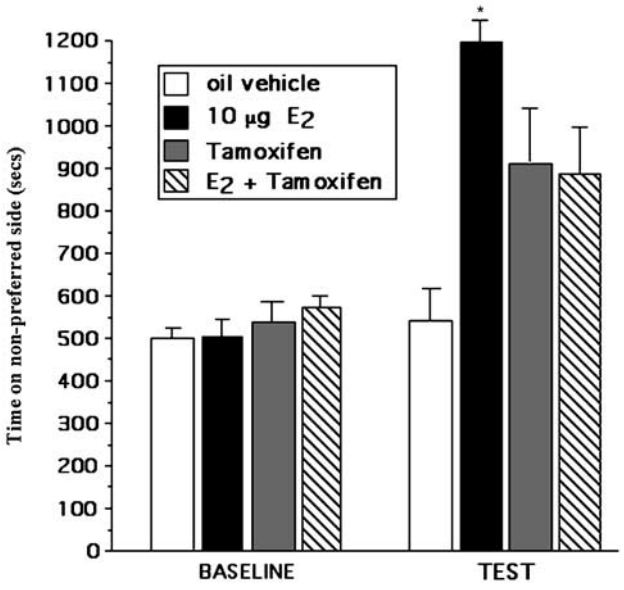

Figure 1 The mean (±SEM) time spent on the nonpreferred side of the conditioning chamber. *Above bar indicates E_2 increases time spent on the nonpreferred side compared to s.c. oil vehicle administration ($P < 0.05$).

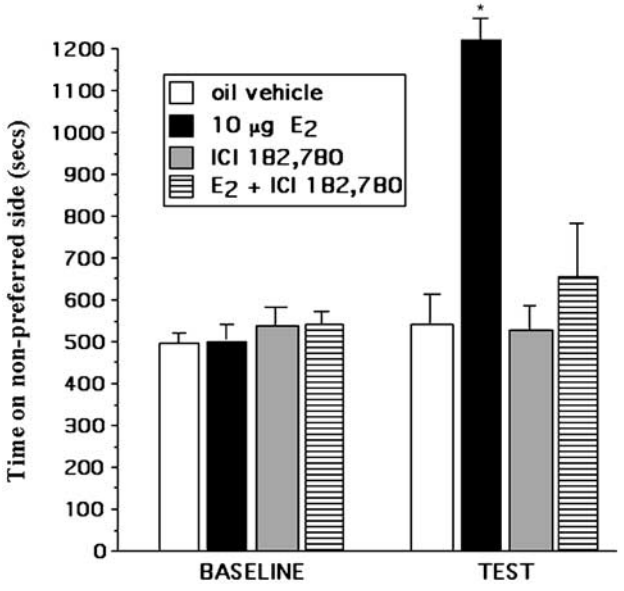

Figure 2 The mean (±SEM) time spent on the nonpreferred side of the conditioning chamber. *Above bar indicates E_2 increases time spent on the nonpreferred side compared to all other groups ($P < 0.05$).

Experiment 2: Effects of ICI 182,780 to the Nucleus Accumbens (Figure 2)

There were main effects of E_2 [$F(1,98) = 12.5$, $P < 0.01$], test occasion [$F(1,98) = 21.6$, $P \leqslant 0.01$], and ICI 182,780 to the nucleus accumbens [$F(1,98) = 6.3$, $P \leqslant 0.01$] and a significant interaction between all three variables [$F(1,98) = 5.4$, $P \leqslant 0.02$]. OVX, E_2-, but not vehicle-, primed rats administered ICI 182,780 spent significantly less time on the nonpreferred side of the chamber during testing than did rats infused with vehicle to the nucleus accumbens.

Experiment 3: Effects of ER Antisense Oligonucleotides to the Nucleus Accumbens (Figure 3)

There was a main effect of infusion condition [$F(2,22) = 5.0$, $P \leqslant 0.01$] and test occasion [$F(1,22) = 22.2$, $P \leqslant 0.01$] on place preference of E_2-primed, OVX rats. Rats administered E_2 and infused with ER antisense oligonucleotides to the nucleus accumbens spent significantly less time on the nonpreferred side of the chamber during testing than did E_2-primed rats infused with scrambled antisense oligonucleotides or vehicle.

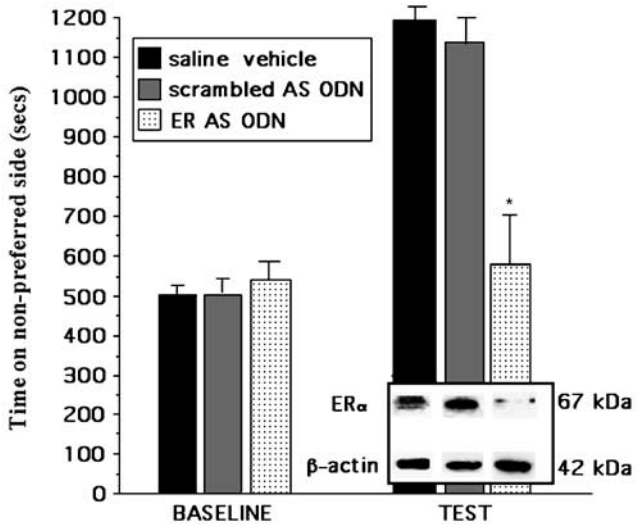

Figure 3 The mean (±SEM) time spent on the nonpreferred side of the conditioning chamber. *Above bar indicates E_2 increases time spent on the nonpreferred side compared to intranucleus accumbens saline vehicle or scrambled control antisense oligonucleotide administration ($P<0.05$). Inset depicts Western blot analyses of ERα and actin protein expression in the nucleus accumbens of these groups.

Efficacy of antisense oligonucleotide infusions is revealed by Western blot analyses. Western blots using the β-actin antibody revealed the presence of protein in samples. Compared to the administration of vehicle or scrambled control to the nucleus accumbens, bands for ERα in nucleus accumbens were not apparent (see Figure 3, inset), demonstrating that ERs were knocked down 24 h after s.c. E_2 administration and ERα antisense oligonucleotides infusion.

DISCUSSION

The present results supported our hypothesis that E_2's effects to produce a CPP may be due in part to actions at ERs in the nucleus accumbens. S.c. administration of E_2 increased time spent on the originally nonpreferred side of the chamber and this was consistently attenuated with coadministration of ER blockers. First, s.c. tamoxifen decreased time spent on the nonpreferred side of the chamber in rats coadministered E_2, but also produced intrinsic effects when coadministered with vehicle. Second, administration of the specific ER antagonist, ICI 182,780, to the nucleus accumbens significantly decreased the time spent on the nonpreferred side of the chamber of rats administered E_2 and did not produce intrinsic effects. Third, administration of ER antisense oligonucleotides to the nucleus accumbens of OVX, E_2-primed rats significantly decreased time on the nonpreferred side and ERα expression in the nucleus accumbens compared to the administration of saline vehicle or scrambled control antisense oligonucleotides. Together, these data suggest that the ability of E_2 to produce a place preference may require actions at ERs in the nucleus accumbens.

The present data confirm previous studies on how E_2 may alter aspects of reward and have intrinsic rewarding effects. Here, we demonstrate that s.c. E_2 administration to OVX rats increases time spent on the originally nonpreferred side of the CPP chamber, which replicates data from a previous experiment. We have previously shown that there are regimen-dependent effects of E_2 to OVX rats to produce CPP, which occur with dosages that produce physiological levels of E_2 in the nucleus accumbens, such as the 10 μg regimen utilized here (Frye and Rhodes, 2006). These findings complement those from other models that demonstrate E_2 also increases performance in other behavioral assays that are typically utilized to assess the rewarding properties of a substance, such as intracranial self-stimulation and self-administration. Naturally receptive and OVX, E_2-administered rats have increased rates of intracranial self-stimulation than do rats with lower E_2 levels (Steiner *et al*, 1982; as reviewed by Carroll *et al*, 2004). Hamsters intracerebroventricularly self-administer E_2 (Dimeo and Wood, 2006). Furthermore, the present study extends these findings of the reinforcing effects of E_2 to suggest that actions at ERs in the nucleus accumbens may be important for E_2-mediated effects on reward.

Systemic administration of the mixed ER agonist–antagonist, tamoxifen, attenuated E_2-induced place preference and enhanced place preference in rats administered vehicle, suggesting that tamoxifen had some intrinsic effects on reward. Tamoxifen, like other selective ER modulators (SERMS), can have both agonistic and antagonistic actions at ERs, depending upon tissue target. This may explain why systemically administered tamoxifen (which could have actions throughout the brain) did not completely block E_2-induced CPP and produced modest increases in place preference when coadministered with vehicle in the present study. Similar patterns of results have been shown previously with systemic tamoxifen administration. Tamoxifen demonstrated E_2 mimetic effects in other systems. For example, systemic tamoxifen has effects similar to E_2 to increase NMDA receptors in the brain (Cyr *et al*, 2001). In other situations, tamoxifen attenuates the effects of E_2. Tamoxifen administered s.c. attenuates antianxiety behavior of OVX rats administered E_2 or SERMs specific to ERβ (Walf and Frye, 2005b). As well, coadministration of E_2 and tamoxifen via s.c. injections attenuated E_2's effects on cocaine self-administration (Lynch *et al*, 2001). There are also scenarios when there is no effect of tamoxifen. For example, tamoxifen is ineffective in altering changes in dopamine release in the striatum or pacing behavior of OVX, E_2-primed rats (Xiao *et al*, 2003). Together, these data suggest that administration of ER ligands, such as E_2 and tamoxifen, can alter reward. However, whether ERs in the nucleus accumbens were required for effects of E_2 on reward were unclear, so this was further examined using more specific pharmacological tools.

Administration of the specific ER antagonist, ICI 182,780, to the nucleus accumbens attenuated E_2-induced CPP, without producing intrinsic effects on CPP. Other studies have demonstrated that ICI 182,780 has functional effects to attenuate behaviors modulated by E_2. ICI 182,780, but not tamoxifen, when administered to the dorsolateral striatum, blocks E_2-facilitated pacing behavior (Xiao *et al*, 2003). Additionally, administration of a specific ER antagonist, ICI 182,780, to the hippocampus increases anxiety and depression behavior of naturally receptive rats (Walf and Frye,

2006). Although our results suggest that intracellular ERs are important, it is not entirely clear whether ICI 182,780 has antagonist effects at membrane and/or intracellular ERs (Gu *et al*, 1999). In addition to actions at intracellular ERs, E_2 can have rapid actions via membrane ERs or other membrane-bound receptors that initiate signal-transduction pathways (Beyer *et al*, 2002; Kelly and Levin, 2001; Moss and Gu, 1999; Nilsen *et al*, 2002; Toran-Allerand *et al*, 2002; Wade *et al*, 2001; Watters *et al*, 1997). Furthermore, it has been postulated that some of E_2 effects at intracellular ERs are potentiated subsequent to the activation of membrane-associated ERs (Kow and Pfaff, 2004; Vasudevan *et al*, 2001). The nucleus accumbens expresses both ER isoforms; however, relatively low levels of intracellular ER mRNA or protein expression in the nucleus accumbens have been demonstrated (Shughrue *et al*, 1997, 1998; Shughrue and Merchenthaler, 2001). The expression of membrane ERs in this region is not clear and it may be that effects of E_2 in the nucleus accumbens are owing to an interaction of membrane and nuclear ERs. The design of the present experiment does not allow us to determine if E_2's effects were rapid (ie occurring in less than 10–15 mins; Pfaff and McEwen, 1983) and through actions involving membrane receptors, but this could be addressed in future studies.

The third strategy that was utilized in the present experiments to investigate E_2's ER-mediated effects for reward was knockdown of ERs with infusions of ER-specific antisense oligonucleotides. We found that administration of ERα antisense oligonucleotides to the nucleus accumbens produced significantly shorter time spent in the non-preferred side of the chamber than did administration of vehicle or scrambled control antisense oligonucleotides. Another study has shown that a similar regimen of ERα antisense oligonucleotides via a single infusion to neonatal rats produced specific behavioral and morphological effects in adulthood (McCarthy *et al*, 1993). In the present study, there was no evidence for nonspecific behavioral effects of the antisense oligonucleotides, based upon neurological testing, which may have obviated effects of E_2 for CPP. Another aspect of this experiment to consider is the timing of the effects. Based upon previous studies utilizing similar antisense oligonucleotides regimen, protein knockdown occurs between 12 and 48 h following antisense oligonucleotide administration (McCarthy *et al*, 1994, 2000; Ogawa *et al*, 1994). Although we cannot know that, at the initial time of conditioning of E_2 and ER antisense oligonucleotides (30 min after administration), expression of ERs was knocked down, knockdown of ERs at 24 h is consistent with the actions of E_2 at ERs being blocked by ER antisense oligonucleotides on the second day in the nonpreferred side of the CPP chamber and Western blot analyses.

The present study, using three strategies to block ERs, suggests that actions of E_2 to produce a CPP may be dependent on activating intracellular ERs in the nucleus accumbens. However, these data need to be interpreted with some caution. There are limitations to the CPP paradigm as a model for addictive behaviors in humans (Bardo and Bevins, 2000; Ettenberg, 2004), such as modest face validity and the question of whether this is a task that measures conditioning to context rather than drugs' interoceptive effects. Future studies could use other models of reward to investigate the mechanisms of E_2 for this effect further. Furthermore, there are potential differences in E_2's effects in this paradigm owing to timing of treatment or regimen utilized. In this study, we have investigated short-term, acute treatment with E_2. For instance, short-term effects of E_2 for affective behavior are not the same as that seen with with longer treatment and/or higher dosing regimen (Luine *et al*, 1998; Morgan and Pfaff, 2001, 2002; Walf and Frye, 2006). Indeed, E_2 produced place aversion and disrupted CPP, when high and/or chronic dosages were utilized (de Beun *et al*, 1991; Galea *et al*, 2001). Thus, the effects of E_2 on behavior are mitigated by many factors, such as timing and dosage of E_2 utilized, which need further investigation to determine the effects of E_2 for chronic conditions, such as mood and anxiety disorders.

The importance of E_2 acting at ERα *vs* ERβ needs to be addressed further. The pharmacological compounds used to block ERs vary in their specificity for blocking these isoforms. Although all blockers attenuated the effects of E_2, it is not clear if ICI 182,780 is specific for ERα or ERβ, there is suggestion that tamoxifen's antagonistic, but not agonistic, effects are through actions involving ERβ (Watanabe *et al*, 1997) and the antisense oligonucleotides strategy was targeted toward ERα. However, these ER isoforms can interact for their functional effects (Lindberg *et al*, 2003; as reviewed by Toran-Allerand 2004), suggesting that the possibility remains that either isoform may be important. Indeed, studies investigating the putative roles of ERα and ERβ for cognitive processes, arousal, and affect suggest the importance of these ER isoforms. Studies utilizing SERMs or ER knockout mice support a role of both ERα and ERβ for learning and memory processes (Fugger *et al*, 2000; Luine *et al*, 2003; Rhodes and Frye, 2006; Walf *et al*, 2006) and ERα for behavioral arousal (Ogawa *et al*, 2003; Pfaff *et al*, 2002). Studies utilizing knockout mice or ERβ SERMs suggest that E_2's actions at ERβ may be important for its antianxiety and antidepressant-like effects. ERβ knockout mice have increased anxiety (Krezel *et al*, 2001), and do not have reduced anxiety or depression behavior in response to E_2 administration, as do their wild-type counterparts (Imwalle *et al*, 2005; Rocha *et al*, 2005; Walf and Frye, 2006). S.c. administration of ERβ, but not ERα, SERMs decrease anxiety and depressive behavior of OVX rats (Lund *et al*, 2005; Walf and Frye, 2005b; Walf *et al*, 2004). Some of E_2's effects on reward processes and behavioral responses to drugs of abuse may be through interactions with the mesolimbic dopaminergic system (as reviewed by Becker, 1999) and ERβ. A recent report suggests that an ERβ-specific SERM produces similar effects as does E_2 to increase D_2 receptor density in the striatum and nucleus accumbens (Le Saux *et al*, 2006). Thus, further investigation of E_2's actions at ERα and/or ERβ and integration with the mesolimbic dopamine system for reward are warranted.

An interesting question is what other modulators, besides putative actions through ERs and/or the mesolimbic dopamine system, may underlie the effects of E_2 for reward and/or affective behaviors. One possibility is that some of E_2's effects on reward and affective processes may be mediated by actions of the hypothalamic pituitary adrenal axis (HPA). Although E_2's effects on glucocorticoid release among female rodents may be regimen dependent (Walf

and Frye, 2005a), there is evidence that E_2 administration increases corticosterone levels of rats administered cocaine (Niyomchai *et al*, 2005). Notably, studies investigating sex differences in relapse rates of drug users suggest that women are more likely to report relapse as a response to stressor exposure (Swan *et al*, 1988; Gritz *et al*, 1996; Snow and Anderson, 2000). Future studies should investigate how the HPA may contribute to the effects of E_2 through actions involving ERs on reward and affective processes.

In summary, these data suggest that E_2's actions to condition a place preference are due in part to actions at ERs in the nucleus accumbens. E_2 administered s.c. produces a place preference in OVX rats. Administration of compounds that block ERs, administered systemically or to the nucleus accumbens, attenuated E_2-induced place preference. It is important to further investigate the sites and mechanisms of action for E_2 as a potential modulator of reward and affective processes.

ACKNOWLEDGEMENTS

This research was supported by a contract grant from the Lilly Centre for Women's Health at Eli Lilly and Company.

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