ACUTE ELEVATION IN ESTRADIOL INFLUENCES THE SALIENCE OF COCAINE CUES

by

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ABSTRACT

ACUTE ELEVATION IN ESTRADIOL INFLUENCES

THE SALIENCE OF COCAINE CUES

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Previous research demonstrates that women and female rodents are more responsive to environmental stimuli associated with drug reward than males. A growing body of literature supports a role for estradiol as one of the mechanisms underlying sex differences in the behavioral response to drugs of abuse. However, little is known about the influence of acute elevations in levels of estradiol on cocaine conditioned behaviors. Thus, the purpose of the present study was to investigate the influence of an acute increase in systemic estradiol levels on the expression of cocaine-induced conditioned place preference (CPP). Experimentally naïve, adult female Long Evans rats were ovariectomized and subjected to the following CPP paradigm: On Day 1, all animals underwent a baseline pretest to determine any pre-existing bias for the two compartments and were then randomly assigned to saline/cocaine conditioning compartments. On Days 2, 4, and 6, animals received an intraperitoneal (i.p.) injection

of 0.9% saline and were confined to their assigned saline-paired compartment for 30 minutes. On Days 3, 5, and 7, they received an i.p. injection of one of three doses of cocaine hydrochloride (0, 5, or 15mg/kg) and were confined to the cocaine-paired compartment for 30 minutes. On the day of the Preference Test (Day 8), all rats received a subcutaneous injection of 5μg 17β-Estradiol 3 benzoate dissolved in 0.1mL of peanut oil (EB) or peanut oil alone (PO) 30 minutes prior to testing. On the day of the preference test, animals were allowed free access to both environments for 15 minutes. The increase in time spent in the cocaine-associated compartment was considered a measure of conditioned preference. Ovariectomized animals treated with PO alone, demonstrated CPP to both doses of cocaine. Interestingly, an acute elevation in estradiol immediately prior to exposure to the previously paired cocaine compartment increased CPP at the high cocaine dose (15mg/kg), but decreased CPP at the lower dose of cocaine (5mg/kg). All animals were sacrificed immediately following the preference test and processed with immunohistochemistry. The results revealed that EB treated females conditioned to 5mg/kg had fewer positive pCREB counts compared to EB females conditioned to 15mg/kg. EB treated females, previously conditioned to 15mg/kg doses of cocaine, had significantly more pCREB positive cells than PO treated females at the same dose.

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INTRODUCTION

1.1 Cocaine use and abuse

Cocaine is a highly addictive psychostimulant. A recent report from the 2010 National Survey on Drug Use and Health revealed that approximately 637,000 persons aged 12 or older used cocaine for the first time within the past 12 months (NSDUH, 2007). These numbers average to approximately 1,700 new users per day or 5% of the population (NSDUH, 2007). Short-term cocaine use induces strong feelings of euphoria, increased energy, and mental alertness in users. While these effects are short-lived and often subside within minutes, long-term use can lead to dependence and addiction. In addition, chronic use of this drug frequently leads to devastating health, legal, and social consequences. Thus, a significant proportion of Americans are current cocaine users and are either at risk for developing or have already developed an addiction, and are in need of treatment.

1.2 Sex differences and patterns of cocaine use

A growing body of epidemiological evidence suggests that, compared to men, women initiate cocaine use at an earlier age, progress from casual cocaine use to cocaine dependence more rapidly, experience higher levels of craving and relapse during periods of abstinence, and take larger amounts of cocaine during bouts of relapse

(Brady & Randall, 1999; Chen & Kandel, 2002; Gallop et al., 2007; Ignjatova & Raleva, 2009; Robbins, Ehrman, Childress, & O'Brien, 1999). Moreover, between the years 2004 and 2009, cocaine was the most commonly identified drug among women during drug-related emergency room visits (accounting for 54% of drug-related emergency department visits among women). Despite this evidence, the majority of the investigations of cocaine dependence and addiction have historically focused on men and male animals.

Animal studies provide further insight into sex differences in the behavioral and motivational responses to cocaine. In particular, rodent studies indicate that the subjective effects of cocaine are more robust in females compared to males (Chin et al., 2002; Forgie & Stewart, 1994). In studies examining cocaine reinforcement and self-administration behavior, female rats acquire intravenous self- administration of cocaine more quickly and at lower doses than males (Davis, Clinton, Akil, & Becker, 2008; Lynch & Carroll, 1999). Furthermore, females administer nearly double the number of cocaine infusions compared to males during the maintenance phase of self-administration (Carroll, Lynch, Roth, Morgan, & Cosgrove, 2004; Lynch, Roth, & Carroll, 2002; Lynch, Roth, Mickelberg, & Carroll, 2001). These results indicate that females may be more sensitive to the reinforcing properties of cocaine and these effects are linked to increases in cocaine intake with continued use. Conditioned place preference experiments from our laboratory have shown that compared to males, females demonstrate an increased preference for cocaine paired environments and a

greater magnitude of reinstatement of conditioned place preference for cocaine after a period of abstinence (Bobzean, Dennis, Addison, & Perrotti, 2010). Taken together, these data support the notion that sex influences motivation to obtain and use cocaine and therefore plays a role in the development of dependence and addiction.

1.3 Interactions between ovarian hormones and the effects of cocaine

Not surprisingly, sex differences in dependence and addiction are due, in part, to the effects of the ovarian hormones estrogen and progesterone. These hormones surge in fluctuating patterns to create the menstrual cycle in humans and estrous cycle in rodents (See Figure 1,Staley & Scharfman, 2005). Clinical studies show that a woman's response to cocaine will vary with her menstrual cycle. Women report having greater positive subjective responses ("feel good rating") to cocaine during the follicular phase of the menstrual cycle, when levels of estrogen are rising and progesterone levels are minimal (Evans & Foltin, 2004, 2006a; M Sofuoglu, Dudish-Poulsen, Nelson, Pentel, & Hatsukami, 1999). In the luteal phase, when progesterone levels are highest (estrogen levels are also elevated at this time), women report reduced positive subjective effects of cocaine (Evans & Foltin, 2004, 2006b; M Sofuoglu et al., 1999). Moreover, exogenous administration of progesterone attenuates some of the physiological and positive subjective effects of cocaine and exacerbates some of the negative subjective effects of the drug (Evans & Foltin, 2006a; Sofuoglu, Mitchell, & Kosten, 2004).This evidence suggests that women's responses to cocaine are strongly influenced by cyclic

hormonal changes and that they may be more likely to engage in drug seeking behaviors when estrogen levels are high and progesterone is low.

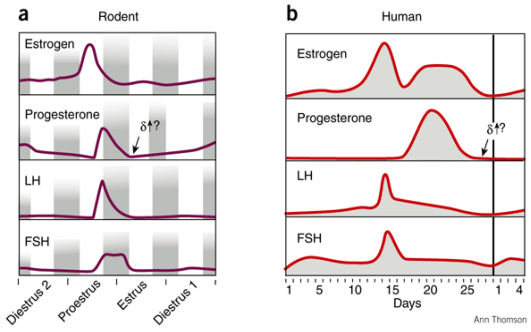


Figure 1. Comparison of rat estrous cycle and human menstrual cycle. (a). the 4 day estrous cycle of the rat, showing estrogen and progesterone, lutenizing hormone, and follicle stimulating hormone fluctuations (b). The 28 day menstrual cycle of the human (reprinted without permission from Staley & Sharfman, 2005)

The above clinical findings are complemented by rodent studies in which levels of ovarian hormones are manipulated. Results from such behavioral studies repeatedly demonstrate that removal of the endogenous source of ovarian hormones via ovariectomy (OVX) decreases cocaine self-administration (Frye, 2007; Larson, Roth, Anker, & Carroll, 2005; Lynch et al., 2001) and replacement of estradiol with chronic subcutaneous injections or implant restores cocaine self-administration rates to levels comparable to intact females' (Becker, 2000; Becker, 1990; Pasqualini, Olivier, Guibert, Frain, & Leviel, 2002; Xiao & Becker, 1994). In summary, results from these experiments demonstrate that ovarian hormones influence responding for cocaine and

may modulate the motivational aspects of cocaine reward. Together, the preclinical and clinical findings indicate that the reinforcing effects of cocaine may be strongly influenced by fluctuations in levels of estrogen and progesterone throughout the reproductive cycle of the female.

1.4 Influence of cocaine on dopamine receptor mediated intracellular responses

Repeated administration of cocaine causes molecular neuroadaptations in the brain that trigger changes in behavior aimed toward obtaining more of the drug (Anderson & Pierce, 2005; Thomas, Kalivas, & Shaham, 2008). The key neural substrate in which these changes occur is in the mesocorticolimbic dopaminergic (DA) system. The mesocorticolimbic DA system is comprised of cell bodies that originate in the ventral tegmental area (VTA) and project to the prefrontal cortex, nucleus accumbens (NAcc), dorsal striatum, amygdala, and bed nucleus of stria terminalis. Dopaminergic transmission within this circuit plays a critical role in modulating the flow of information through the limbic system to mediate motivated behaviors. A great deal of evidence indicates that changes in neural activity within the mesocorticolimbic DA system underlie drug reward and contribute to relapse and cocaine seeking behavior (P. W. Kalivas & McFarland, 2003; R C Pierce & Kalivas, 1997; Wise & Bozarth, 1987).

Studies conducted solely in male animals have revealed that cocaine administration affects several DA receptor mediated intracellular responses in the NAcc

including 3”-5”cyclic AMP (cAMP) and protein kinase A (PKA) (Nestler, 2002; Walters, Kuo, & Blendy, 2003). Dopamine receptor activation induces the cAMP response element binding protein (CREB) through a PKA-dependent intracellular mechanism (Brami-Cherrier et al., 2002; Culm, Lugo-Escobar, Hope, & Hammer, 2004; Dudman et al., 2004; R C Pierce & Kalivas, 1997; Yan, Feng, Fienberg, & Greengard, 1999). The phosphorylated (activated) form of CREB (pCREB) regulates the expression of several genes important to brain reward function (McClung & Nestler, 2003). Furthermore, manipulation of CREB levels in the male rodent brain affects the rewarding properties of cocaine (Carlezon et al., 1998; Pliakas et al., 2001; Walters et al., 2003). In this way, CREB activity in the NAcc is implicated in cocaine reward and serves as a regulator of the negative emotional symptoms that occur during cocaine withdrawal (Carlezon Jr, Duman, & Nestler, 2005; Carlezon et al., 1998; Pliakas et al., 2001; Walters & Blendy, 2001).

1.5 Estradiol modulation of dopamine signaling

It is well established that DA activity in the NAcc is modulated by the estrous cycle (Becker, 1999; Lynch et al., 2002; Perrotti et al., 2006; Russo, 2003). DA extracellular concentrations, are higher during estrous - when levels of estradiol are high – than during diestrus. Striatal DA uptake sites are more numerous during proestrus, and DA-receptor density is higher during estrus and proestrus as compared with the other stages of the cycle (Becker & Cha, 1989; Becker, 1990; Becker, 1999;

Jori & Cecchetti, 1973; Lévesque & Di Paolo, 1989; Xiao & Becker, 1994).

Several studies have demonstrated the modulation of behavioral and neurochemical activities in midbrain DA systems by estradiol (Becker, 1990; Di Paolo, Rouillard, & Bédard, 1985). For example, an acute injection of physiological doses of estradiol increases striatal DA release and turnover and increases the density of striatal DA uptake sites (Becker & Beer, 1986; Becker & Ramirez, 1981; Di Paolo et al., 1985; Morissette, Biron, & Di Paolo, 1990). These effects of estradiol on striatal DA activity are, in part, responsible for the sex and hormone-related differences in subjective and physiological responses to cocaine (Becker & Ramirez, 1981; Becker, 1999; Quiñones-Jenab, 2006; Walker et al., 2001). However, the underlying mechanism(s) by which estradiol influences the effects of cocaine on neuronal DA systems remain largely unknown.

Midbrain DA systems contain high numbers of estrogen alpha (ERα) and beta receptors (ERβ) (Creutz & Kritzer, 2002, 2004). There is evidence that estradiol regulation of DA D2 type receptors in the striatum occurs through the activation of the ERβ (Morissette et al., 2008). Chronic estradiol administration increases dorsal and ventral striatal D1 receptor density and binding through activation of the ERβ (Becker, 1999; Lévesque & Di Paolo, 1989; Zhou, Cunningham, & Thomas, 2002). Thus, estradiol-induced activation of the ERβ in the NAcc is involved in the up regulation of DA receptors. The up regulation of these DA receptors is critical for reinstatement of drug seeking behavior. In addition, changes in levels of estradiol regulate the firing rate

of DA neurons, cause changes in DA release, and changes in DA D1 receptor density in both the dorsal striatum and NAcc (Becker, 1999; Lévesque & Di Paolo, 1989; Zhou et al., 2002). In this way, estradiol influences DA transmission via indirect effects on D1 receptors.

It is well established that estrogens produce their effects by genomic and non-genomic actions. The so-called "genomic estrogen receptors" are ligand-activated transcription factors which reside in the cytosol and translocate to the nucleus upon ligand binding and dimerization (Nilsson et al., 2001). As with other steroid hormone receptors, ERs can either modulate gene expression directly, by binding to consensus target DNA sequence, or indirectly, by interacting with other transcription factors to activate or repress gene activation.

Estradiol also has acute, rapid (nongenomic) effects, which are initiated via binding at estrogen receptors localized on the cell membrane; membrane estrogen receptors (mERs) (Boulware et al., 2005; Mermelstein & Micevych, 2008; Micevych & Mermelstein, 2008). Signaling at mERs activate G-protein dependent cell signaling cascades (Hammes & Levin, 2007) including PKA and MAPK. The signaling cascades initiated via mERS are some of the same that are initiated by DA at D1 receptors. In fact, evidence for the role for mERs in mediating the rapid effects of estradiol stems from its effects on CREB phosphorylation (pCREB). Figure 2 represents a simplified model to illustrate the parallel signaling pathways initiated via D1 and mER in the striatum. In this way, estradiol activates mER and D1 receptor G protein-dependent cell

signaling cascades including activation of the MAPK pathway, and phosphorylation of CREB (Hammes & Levin, 2007). In light of these findings, research investigating the experimental manipulation of mERs has received increasing attention. In one study, the use of the pure ER antagonist ICI 182,780 blocked the estradiol dependent increase in striatal DA release following an injection of amphetamine (Xiao, Jackson, & Becker, 2003). ICI 182,780 also blocks the rapid effects of estradiol on pCREB. Furthermore, in behavioral experiments, local application of ICI 182,780 into the NAcc reversed estradiol induced increases in return latency in OVX females during paced mating (Becker, 1999). Together, these findings demonstrate that activation of mERs plays a modulatory role in motivational aspects of behavior. However, little is known about the functional significance of mER signaling in the NAcc as it relates to cocaine reward, although much can be speculated from the molecular evidence.

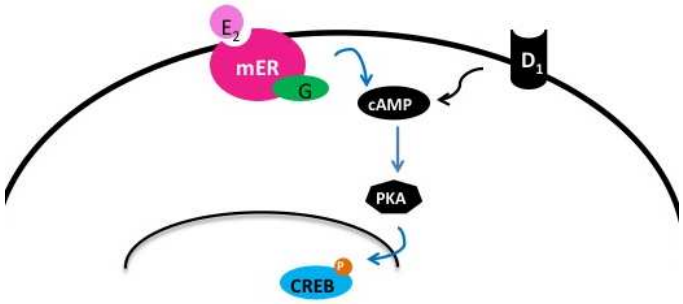


Figure 2. The non-genomic mode of estrogen signaling. Estrogen binds to a membrane estrogen receptor (mER) and activates cAMP and protein kinase within cells rapidly. This triggers nuclear phosphorylation of CREB.

In conclusion, sex differences in motivated drug seeking behaviors are likely regulated by estradiol mediated changes in DAergic signaling which influence cocaine-induced alterations in intracellular signaling cascades. While several studies have demonstrated that estradiol modulates behavioral and neurochemical activities involved in memory formation, associative learning, and responding to reinforcing stimuli following exposure to cocaine (Lynch & Carroll, 1999; Lynch et al., 2001; Russo, 2003), none have fully examined the functional interplay of estradiol and dopamine regulated intracellular signaling in response to cocaine and cocaine conditioned reward. The aim of the following experiments was to build upon previous literature and further characterize the role of estradiol in the behavioral expression of cocaine reward, and investigate the rapid effects of estradiol induced intracellular signaling in the NAcc via measuring pCREB expression immediately after the expression of conditioned place preference for cocaine.

1.6 Purpose and hypotheses

There is a surprising gap in our current understanding of the molecular mechanisms underlying gonadal hormone-dependent changes in reward processing. Further, the direct effects of estradiol on conditioned drug reward are virtually unknown. Thus main objective of the current study was to evaluate the acute activational effects of estradiol on cocaine conditioned reward and intracellular signaling in the NAcc. To date, no studies exist that examine the molecular signaling

adaptations resulting from the interactions between cocaine, conditioned reward and estradiol.

The first hypothesis was that cocaine would dose dependently enhance locomotor activity in OVX female rats as measured by comparing locomotor activity between saline and cocaine conditioning sessions. The second hypothesis was that an acute elevation in estradiol would augment the expression of preference for noncontingent cocaine cues in the OVX female rats, as shown by an increase in time spent in the cocaine paired chamber. The third, and last hypothesis, was that acute estradiol would alter intracellular signaling in the NAcc, as measured indicated by alterations in pCREB expression in the NAcc following the preference test.

CHAPTER 2

METHOD

2.1 Subjects

Fifty-four experimentally naïve, adult, female, Long Evans rats were triple housed with same-sex cage mates in a temperature and humidity-controlled environment under a 12h reversed light/dark cycle with lights on at 7p.m. and off at 7a.m. All animals had free access to food and water throughout the study and were maintained and cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2 Ovariectomy

Adult female rats (60 days old) were anesthetized with a 2-3%isoflurane-oxygen vapor mixture and ovariectomized (OVX) using a dorsal approach. Briefly, both flanks were shaved and swabbed with Betadine. The skin was opened with a 5mm incision along the midline just below the ribs, and a 10mm incision was made through the muscle ~1.5-2cm lateral to the midline. The ovary was pulled through the incision. The tissue between the oviduct and uterus were clamped with a hemostat and a ligature was placed just below the hemostat. The ovary was removed with scissors and the hemostats released. This procedure was repeated on the contralateral side. Lastly, the muscle layer was sutured closed and the skin incision closed with 9mm wound clips.

2.3 Vaginal lavage testing

Following a 4-5 day surgical recovery period, all rats underwent daily vaginal lavage testing daily for 10 consecutive days to confirm cessation of cycling. Vaginal secretion was collected with a plastic pipette filled with 10μL of 0.9% saline (SAL) by inserting the tip into the rat vagina, but not deeply. Unstained material was observed under a light microscope. All ovariectomies performed were confirmed as complete and thus, no animals were eliminated on the basis of an incomplete procedure.

2.4 Hormone treatment

All female rats were assigned to one of two groups of hormone treatment (n = 8-10) per group: 0.1ml peanut oil - vehicle (PO); or 5μg 17β-Estradiol 3 benzoate (EB; Sigma-Aldrich, St Louis, MO) dissolved in 0.1ml peanut oil. Hormone treatment was delivered (subcutaneously) only once on the test day of conditioned place preference), thirty minutes prior to the test.

2.5 Cocaine Conditioned Place Preference

The apparatus used to carry out the conditioned place preference (CPP) consists of a two large chambers distinct in visual and tactile cues (wall color and floor material) that are connected by a small shuttle chamber (Med Associates, Georgia, VT). Behavioral testing began 12-14 days after OVX surgery; following OVX confirmation.

On Day 1 of CPP, all rats underwent a Preconditioning Test allowing them to freely explore the entire apparatus for one 15-minute session. Rats were then randomly assigned to SAL/cocaine conditioning chambers for a total of six conditioning sessions. On each of the three SAL conditioning days, animals received a 1ml/kg intraperitoneal (i.p.) injection of 0.9% SAL and were confined to the SAL-paired chamber for 30 minutes. For each of the three cocaine conditioning days, animals received an i.p. injection of cocaine hydrochloride (5 or 15 mg/kg; Sigma-Aldrich, St Louis, MO) at a volume of 1ml/kg dissolved in 0.9% saline and were placed in the drug-paired chamber for 30 minutes. On the day of the Preference Test, rats were allowed free access to all chambers for 15 minutes. Thirty minutes prior to the Preference Test, animals received a subcutaneous (s.c) injection of 5μg EB in 0.1mL of peanut oil or peanut oil (PO) (see Figure 3 for timeline of procedures). Locomotor activity during conditioning and time spent in each chamber during the pretest and post-test were automatically recorded for subsequent statistical analyses using MedPC software (Med Associates Georgia, VA).

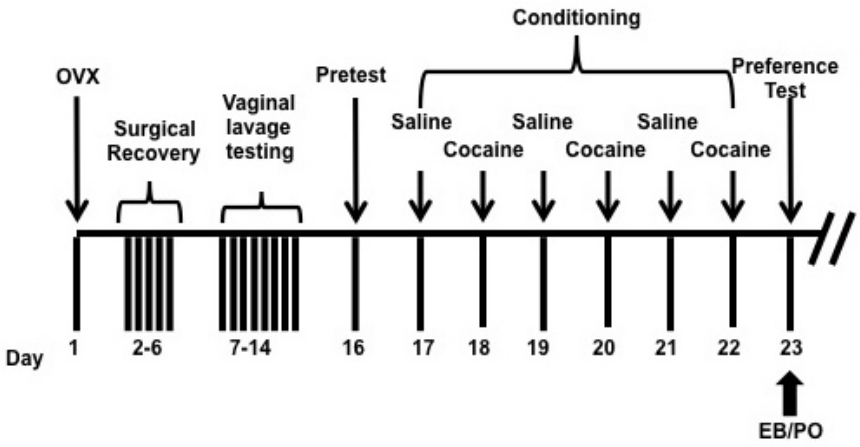


Figure 3. Timeline of experimental procedures: PreTest (Day 1): rats freely explore the apparatus in a drug-free state. Saline-Cocaine conditioning took place on days 2-7; on alternating days rats received injections of saline and were confined to one side of the apparatus for 30 minutes. The following day they received cocaine (0, 5, or 15mg/kg) and were confined to the opposite side of the apparatus. Thirty minutes prior to the preference test (Day 8), animals received a s.c. injection of EB (5μg) or PO.

2.6 Immunohistochemistry

2.6.1. Tissue Fixation

Immediately following the test, animals were deeply anesthetized with a single 1.0ml i.p injection of chloral hydrate (400 mg/ml dissolved in 0.9% saline; Sigma Aldrich, St. Louis, MO) and perfused transcardially with 200ml of ice cold 0.01M PBS followed by 400ml of 4% paraformaldehyde made in 0.01 M PBS. Brains were removed, placed in 4% paraformaldehyde and stored at 4˚C overnight. The next day, brains were placed in 20% glycerol for postfixation for a minimum of 24 hr.

2.6.2. Sectioning and Staining

Brains were sectioned (40 µm thick) on a freezing microtome (MICROTOME 411) and coronal sections stored in 0.01% sodium azide (Sigma Aldrich, St. Louis, MO) dissolved in 1M PBS at 4°C. Sections from the NAcc were selected from each brain and stained immunohistochemically for pCREB using previously described methods (Elmquist et al., 1996). Briefly, tissue was rinsed with 3% hydrogen peroxide (Sigma Aldrich, St. Louis, MO) in order to destroy any endogenous peroxidases. Next, sections were blocked for one hour with 3% normal donkey serum (Jackson ImmunoResearch, West Grove, PA) and 0.3% Triton X (Sigma Aldrich, St. Louis, MO) followed by incubation in rabbit anti-pCREB antiserum (1:1500; Millipore Billerica, MA) for approximately 20 hr with 1% normal donkey serum. A biotinylated donkey anti-rabbit secondary antiserum (Jackson ImmunoResearch, West Grove, PA) was used at a

dilution of 1:200. Tissue was then incubated with avidin-biotin complex (Vectastain ABC Elite kit; Vector Laboratories, Burlingame, CA) for 1.5 hrs, and pCREB immunoreactive (IR) nuclei were visualized by reaction with 3,3'-diaminobenzidine (DAB; Vector Laboratories Burlingame, CA), 3% H_2O_2, and 0.01%$CoCl_2$. After staining, sections were rinsed in PBS, mounted onto positively charged microscope slides, and cover-slipped prior to cell counting.

2.6.3. Cell counts

Two persons blinded to treatment conditions were responsible for all counts using a Zeiss axioimager. pCREB immunoreactive nuclei of the NAcc were counted in sections on both sides of the brain. A total of 4 quantifications were completed per animal for each counter. These quantifications were then averaged, compared, and reported in the final results.

2.7 Statistical Analyses

2.7.1. Locomotor Activity

It was hypothesized that cocaine would dose dependently enhance locomotor activity in OVX female rats as measured by comparing ambulatory activity between saline and conditioning sessions. To test this hypothesis a 3 (Dose) x 6(Conditioning) mixed Analysis of Variance (ANOVA) was conducted and animals' ambulation during all six conditioning sessions were compared by post-hoc analyses with Fisher's least

significant differences.

2.7.2. Conditioned Place Preference

Additionally, it was hypothesized that an acute elevation in estradiol would augment conditioned responding for cocaine reward in the ovariectomized female rats, as shown by higher preference for noncontingent cocaine cues. To test this hypothesis, a 3 (Dose) X 2 (Treatment) X 2 (Time) mixed ANOVA was conducted to examine the effects of EB and Cocaine on preference scores. Statistically significant interactions were followed by post-hoc analyses with Fisher's least significant differences.

2.7.3. pCREB

Lastly, it was hypothesized that acute estradiol would alter neurobiological signaling during the preference test, as shown by increased pCREB expression in the NAcc of EB treated females. To test this hypothesis, a 3 (Dose) X 2 (Treatment) mixed ANOVA was conducted to examine the effects of EB on pCREB expression. Statistically significant interactions were followed by post-hoc analyses with Fisher's least significant differences.

CHAPTER 3

RESULTS

3.1 Locomotor Activity

Cocaine dose dependently enhanced locomotor activity throughout CPP conditioning $F(10, 78) = 3.13$, $p < .01$, *partial* $\eta^2 = .29$. Only females conditioned to 15mg/kg of cocaine exhibited significantly increased locomotor activity during cocaine conditioning sessions compared to saline conditioning sessions. There were no differences in locomotor activity between cocaine conditioning sessions and saline conditioning sessions in OVX females conditioned to 5mg/kg of cocaine or those that received saline throughout all conditioning days. (See Figure 4 for summary.)

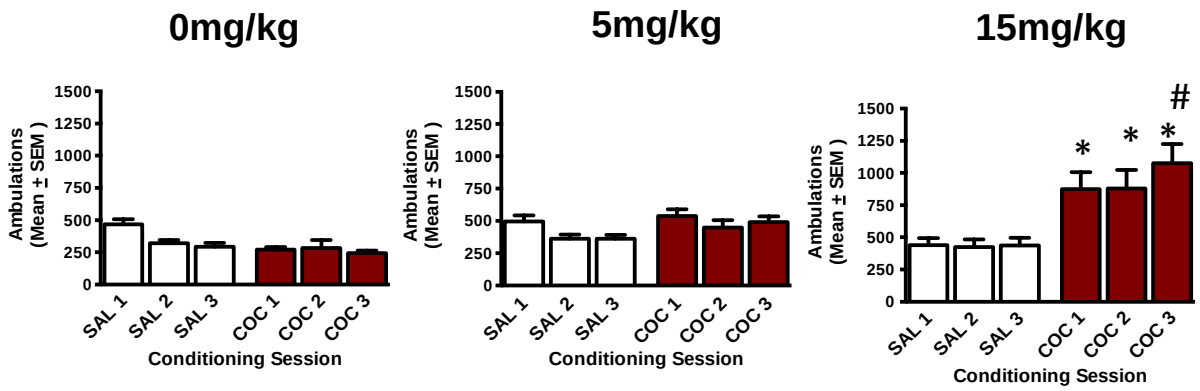


Figure 4. Locomotor activity over the three saline and three cocaine conditioning sessions in OVX females (prior to PO or EB treatments). Locomotion is measured as two consecutive beam breaks during one 30 minute conditioning session in each of the saline paired (white) or cocaine paired (red) chambers. * Indicates a significant difference from saline ($p < 0.05$). # indicates significantly different cocaine from 5mg/kg conditioning sessions ($p < 0.05$).

3.2 Conditioned Place Preference

No significant main effect of treatment was detected, but there was a significant main effect for Dose, $F(2, 39) = 3.47$, $p = .04$, *partial* $\eta^2 = .15$ and a main effect of Time, $F(1, 39) = 14.64$, $p < .001$, *partial* $\eta^2 = .27$. Most importantly, there was a Dose X Treatment X Time interaction $F(2, 39) = 4.79$, $p = .01$, *partial* $\eta^2 = .20$. This significant interaction was followed by post-hoc tests to assess if CPP scores during the Post-test were different within each dose and treatment tested. Post hoc comparisons for each dose of cocaine tested revealed that PO treated females preferred the formerly cocaine paired chamber for 5mg/kg ($M = 149.58$, $SE = 61.35$; $p < .01$) and showed marginal preference for 15mg/kg ($M = 123.75$, $SE = 61.35$; $p = .068$). EB treated females rats however, demonstrated preference for the cues paired with 15mg/kg ($M = 245.27$, $SE = 61.35$; $p < .01$) dose of cocaine but not 5mg/kg ($p = .41$). (See Figure 5 for summary.)

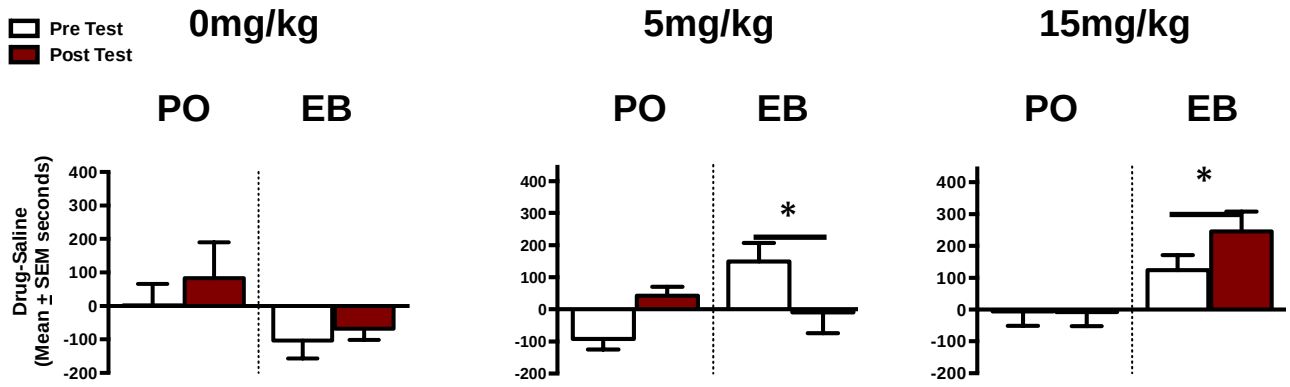
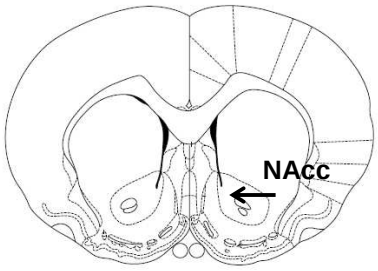


Figure 5. Cocaine-induced CPP (0, 5, and 15mg/kg) in OVX rats treated with EB (5ug; s.c.) or PO 30 minutes prior to the PostTest. Preference data are expressed as a CPP score values were obtained by subtracting the total time spent in the cocaine paired chamber from the total time spent in the saline paired chamber during the PreTest (white bars) and Post Test (red bars). Non-estradiol treated animals (PO) expressed a marginal preference to 5 ($p = 0.068$) and a significant preference to 15mg/kg ($p < 0.05$) of cocaine compared to the pretest CPP score). EB treated animals expressed a preference only to the 15mg/kg conditioning dose of cocaine ($p <$ .05; compared to the EB pretest CPP score).

3.3 pCREB

No main effects for treatment or dose were observed. However, there was a significant Dose X Treatment interaction $F(2, 26) = 13.54$, $p < .01$, *partial* $\eta^2 = .509$. PO treated females conditioned to saline ($M = 930.58$, $SE = 76.40$) and 5mg/kg ($M = 881.96$, $SE = 50.02$) had more positive pCREB counts compared to PO females conditioned to 15mg/kg. EB treated females conditioned to 5mg/kg ($M = 744.71$, $SE = 54.02$) had fewer positive pCREB counts compared to EB females conditioned to 15mg/kg ($M = 1005.90$, $SE = 59.18$). EB treated females ($M = 1005.90$, $SE = 59.18$), previously conditioned to 15mg/kg doses of cocaine, had significantly more pCREB positive cells than PO treated females ($M = 601.76$, $SE = 54.03$) at the same dose. No other significant pairwise comparisons were found. (See Figure 6 for summary.)

A



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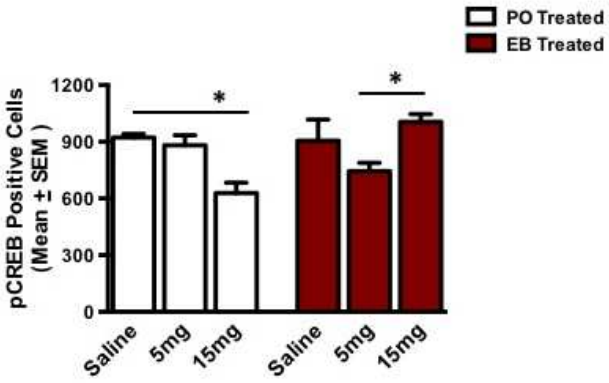

Figure 6. pCREB expression in the NAcc after cocaine CPP. **A,** Area where cells were quantified. **B** pCREB positive cell number quantification in animals receiving PO (white bars) or EB (red bars) 30 minutes prior to the Post Test. Note that pCREB levels after 15mg/kg CPP PostTest are significantly higher in the NAcc of EB-treated rats compared to PO treated rats ($p < 0.05$).

CHAPTER 4

DISCUSSION

Cues and cue reactivity are important in drug addiction, and may be particularly important in women. While previous experiments mostly examine the effects of chronic estradiol treatments on reactivity to cocaine conditioned cues, the literature supports that estradiol has specific rapid effects that may temporarily/periodically increase woman's responsivity to cocaine associated cues. It is therefore important to further characterize the effects of acute elevations in estradiol on behavioral output and neurobiological signaling. The current study was designed to investigate the acute effects of estradiol on reactivity to cocaine conditioned cues using a conditioned place preference paradigm, and to identify changes in phosphorylated CREB expression in the NAcc at the time of preference testing in female rats. We hypothesized that estradiol treated females would demonstrate increased CPP for cocaine and that pCREB expression in the NAcc of estradiol treated rats demonstrating CPP for cocaine would be enhanced compared to non-estradiol treated animals also demonstrating CPP for cocaine and estradiol treated control animals. The major findings of this study are 1) Cocaine dose dependently enhances locomotor activity in OVX female rats 2) estradiol modulates salience for environments previously paired with cocaine in a manner dependent on the conditioning dose; and 3) induction of pCREB expression in the NAcc is influenced by acute elevations in estradiol and during CPP.

4.1 Locomotor Activity

Exposure to psychostimulants such as cocaine causes changes in rodent locomotor activity (Kalivas, Duffy, DuMars, & Skinner, 1988; R.Christopher Pierce & Kalivas, 1997; Post and Rose, 1976). The results of the present experiment demonstrate that cocaine dose dependently increased locomotor activity during cocaine conditioning in OVX female rats. There were no significant changes in the magnitude of locomotor activity between cocaine-conditioning sessions for any of the doses tested. Although the present experiments were not designed to investigate the role of estradiol on conditioned locomotor responses, the data are in line with previous findings that cocaine dose and ovarian hormones mediate certain behavior enhancing properties of cocaine in female rats (Glick, Hinds, & Shapiro, 1983; Van Haaren & Meyer, 1991). Specifically, removal of ovarian hormones by OVX has been shown to decrease behavioral responses for low doses of cocaine (Hu & Becker, 2003). Such research supports the present finding that only the high conditioning dose of cocaine significantly enhanced the magnitude of locomotor activity compared to saline conditioning days in OVX rats. Further experiments are necessary in order corroborate the reversing effects of estradiol replacement on OVX attenuation of cocaine-induced behavioral sensitization in female rodents. Such experiments will elucidate the facilitatory role of estradiol on sensitivity to the conditioning effects of cocaine in females.

4.2 Conditioned Place Preference

The results further demonstrate that acute systemic elevations in estradiol influence preference for conditioned cocaine reward in female rats; inhibiting the expression for a low conditioning dose, but having little effect on preference for a high conditioning dose of the drug. In contrast, previous studies manipulating circulating levels of estradiol in rodents have consistently demonstrated a key role for estradiol in *enhancing* the behavioral response to cocaine in females (Becker, 1990; Becker, 1999; Thompson, 1999). For example, removal of ovarian hormones by OVX decreased acquisition rates of cocaine self-administration and cocaine primed reinstatement of drug seeking behavior; replacement of estradiol via daily chronic subcutaneous injections or Silastic implant, restores cocaine self-administration rates to levels comparable to intact females' (Larson, Anker, Gliddon, Fons, & Carroll, 2007; Lynch et al., 2001). Interestingly, the majority of previous investigations that have demonstrated the importance of elevations of estradiol in enhancing the behavioral responses to cocaine have utilized chronic, intermittent, or continual estradiol administration regimens. These regimens result in high and/or sustained estradiol circulating levels of estradiol for several consecutive days, which creates an unnatural hormonal milieu for the animal. The current literature is lacking in studies in which dose or length of estradiol administration treatment is varied. The few that do exist, demonstrate such variations in treatment have drastic effects on the behavioral response to cocaine. For example, one such study demonstrated that chronic injections of physiological doses of

estradiol given to OVX rats resulted in more avid responding for cocaine than a higher dose of estradiol (Hu & Becker, 2008).

Previous research (Perrotti et al., in preparation) using this animal model of Pavlovian conditioning has demonstrated that chronic estradiol treatment alters conditioned place preference for cocaine in OVX female rats. Specifically, OVX females treated daily with estradiol have enhanced cocaine CPP scores compared to PO treated females. Interestingly, the results from the present study demonstrate that OVX females treated acutely on the day of preference test with estradiol also demonstrated cocaine CPP but the magnitude of preference compared to PO treated was not significant, as was found in previous experiments. Thus, the differences in results between the present study and previous experiments performed in our lab emphasize the influence of timing of estradiol treatment on the expression of learned behaviors. It appears that the enhancing effects of estradiol on CPP may occur during conditioning by either increasing the rewarding effects of cocaine or by strengthening the associations between cocaine reward and the environmental context in which it was experienced. Further experimentation examining the effects of estradiol given only during the conditioning phase of the CPP paradigm is necessary to fully confirm this new hypothesis.

One explanation for the present findings is that estradiol may be attenuating the recall of the association between associations between cocaine reward and

environmental context. Several studies have shown that estradiol can differentially affect memory processing. For example, some studies have shown that estradiol enhances learning and retention of certain memory tasks, such as the Morris Water Maze (Foster, 2003; Gibbs, 2000; Luine, Richards, Wu, & Beck, 1998). In contrast, reference memory, a necessary process for retrieval of a drug-context association is impaired following estradiol treatment (Galea, 2001). Still, most of these studies used repeated estradiol administration throughout training phases prior to performance tasks. The present study is the first to raise implications of estradiol's acute effects on expression of previous associative learning. Ultimately, the differences between the present study and previous experiments performed in our lab (and others) emphasize the influence of timing of estradiol treatment on the expression of learned behaviors.

4.3 pCREB

Previous research has demonstrated converging parallel intracellular signaling induced by DA and mERs in the NAcc and suggests a common mechanism for regulating nuclear transcription factors. In this way, estradiol activates mER and D1 receptor G protein-dependent cell signaling cascades including activation of the MAPK pathway, and phosphorylation of CREB (Hammes & Levin, 2007). In line with this research, we expected that acute estradiol would alter intracellular signaling in the NAcc, as measured by alterations in pCREB expression in the NAcc following the cocaine CPP. The results of the current study, however, produced somewhat

unexpected results. Within the NAcc, PO treated females conditioned with saline and the low dose of cocaine had a greater number of pCREB positive cells compared to PO-treated rats conditioned with the high dose of cocaine. Contrary to our expectations, an acute elevation in estradiol did not result in increased numbers of pCREB positive cells, as seen in EB treated females conditioned with saline. Interestingly, EB treated females conditioned to the low dose of cocaine had fewer positive pCREB cells compared to estradiol treated females conditioned to the high dose of cocaine.

The seemingly perplexing nature of these results is not completely surprising given the complex nature of ER signaling. Activation of ERs can activate PKA activity, nuclear CREB phosphorylation, and subsequent regulation of gene transcription (reviewed by Micevych & Mermelstein, 2008). Similarly, exposure to cocaine and conditioned cues has also been shown to trigger the same intracellular events (Shiflett, Mauna, Chipman, & Peet, 2010). However, in this study, it is likely that in addition to the direct rapid effects of estradiol on pCREB expression, the findings may also reflect estradiol's indirect influence on dopamine PKA activity during exposure to cocaine cues. This supports the notion that in addition to estradiol, pCREB levels were likely influenced by the secondary rewards of the environmental stimuli of the CPP apparatus following cocaine conditioning. Future studies are needed to parcel out these intracellular events and to help elucidate mechanisms for consequential alterations in behavioral output.

Although acute estradiol administration seemed to play a critical role in

accumbal CREB phosphorylation and expression of CPP, at this time, the exact relationship between estradiol, pCREB expression, and CPP is not entirely clear. In the present experiment, pCREB appeared to fluctuate independently from preference in PO and EB treated females. Interestingly, EB treated females, previously conditioned to 15mg/kg doses of cocaine, had significantly more pCREB positive cells than PO treated females at the same dose, although there were no notable differences in preference at this dose. These results are in direct opposition of previous findings that have demonstrated relatively straightforward relationships between CREB and drug conditioned behavior. In one study, higher pCREB was associated with attenuations in cocaine CPP while low pCREB expression is characteristic of enhanced CPP, however such findings are from studies performed solely in male rodents and in paradigms with direct manipulation of CREB activity via microinjections into the nucleus accumbens (Pliakas et al., 2001). To our knowledge, no other studies have sought to examine the shared regulation of CREB via DA and ER signaling and the effects on cocaine conditioned reward; thus, further investigation is needed.

4.4 Limitations

Methodological limitations of this study are a likely contributing factor to the ambiguity of the present results. First of all, we made no assessment of pCREB prior to CPP testing to offer an indication estradiol's effect on pCREB change following the preference test. Such comparisons would require a more intricate experimental design

and larger sample sizes in order to adequately address this problem. Secondly, it was our contention that acute elevations in estradiol would enhance pCREB expression during the preference test. The nature of the preference test involves allowing the rat to freely explore the entire CPP apparatus. Because of this, the rat is repeatedly transitioning from the cocaine-paired environment to the saline-paired environment. The instability of cue presentation poses a problem for consistent cue induced dopamine signaling between groups of animals. One way to correct for this would be to administer an exposure test following the preference test in which all animals were confined to the cocaine paired chamber prior to sacrifice and subsequent analysis of cue induced CREB phosphorylation. Again, larger sample sizes would be necessary in order to accommodate the expansion of experimental design.

4.5 Concluding Remarks

In summary, the present data show that estradiol plays a crucial role in both the expression of cocaine CPP and has rapid effects on intracellular signaling in the NAcc. This is an important finding since numerous investigations have been dedicated to the role of chronic estradiol in conditioned cocaine reward, whereas the acute rapid effects of estradiol have hardly received any attention. Further characterization of the relationship between cocaine cues, ovarian hormones will lead to a better understanding of the mechanisms that underlie the sex dependent differences in vulnerability to drug addiction behavior.

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