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The role of brain estrogen receptor activation in motivation for cocaine in pregnant Sprague Dawley rats

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THE ROLE OF BRAIN ESTROGEN RECEPTOR ACTIVATION IN MOTIVATION FOR COCAINE IN PREGNANT SPRAGUE DAWLEY RATS

BY

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Cocaine use is prevalent in the United States and can initiate a spiraling series of consequences. Of the 5.2 million people per year who use cocaine in the United States, there are about 750,000 cocaine-exposed pregnancies (*What Are the Effects of Maternal Cocaine Use?*, 2016). Usage during pregnancy creates a unique set of complications given that both the mother and baby are at risk. When taken in by a pregnant woman, cocaine crosses through the placenta and interacts with the fetus leading to an array of medical and developmental defects. In terms of medical concerns for the baby, there is a higher risk of reduced intrauterine growth, development of infections, and central and autonomic nervous system symptoms such as irritability and hyperalertness (Bauer et al., 2005). Later on in life, prenatal exposure to cocaine makes children more likely to experience poor emotional regulation, learning disabilities, and language delays (Behnke et al., 2013). This major focus on outcomes for the drug-exposed fetus has resulted in minimal research specifically examining the mothers and their experience with substance use and addiction during pregnancy. Within this limited data, researchers have looked into trends in the demographics factors associated with pregnant women who use cocaine. Through both single-institution and national survey studies, it has been found that pregnant women who use cocaine are more likely to be unmarried, non-White, and unemployed (Frank et al., 1988) (Havens et al., 2009). There is also evidence of polydrug use where sixty-five percent of sweat patches administered to opiate-dependent pregnant women at the Johns Hopkins Bayview Medical Center detected cocaine, while thirty-five percent of patches were positive for both cocaine and additional opiate drug use (Brunet et al., 2010). These data highlight the patterns that

exist for pregnant women using cocaine and identify possible risk factors or reasons as to why these women may initiate cocaine use.

Pregnant women are susceptible to many adverse health outcomes as a result of using cocaine. Apart from general cocaine use risks, pregnancy can exacerbate these consequences for the mother. For instance, pregnant women with positive cocaine urine analysis tests at the Irvine Medical Center presented with symptoms of headaches, seizures, visual impairment, and high blood pressure (Towers et al., 1993). While these patients were initially presumed to have preeclampsia or eclampsia, doctors diagnosed them with cocaine intoxication given that their hypertension rapidly resolved and blood laboratory measures did not show abnormal results, which is not typically seen in cases of these pregnancy-related conditions. Though this research does not show what percent of cocaine-exposed pregnancies present with these health-related issues compared to typical pregnancies, it demonstrates how exposure to cocaine during pregnancy may be associated with specific negative health consequences in the mother. Comparisons between pregnant and nonpregnant models have been studied in preclinical research. In one study, pregnant and nonpregnant sheep administered various doses of cocaine were assessed for several physiological responses (Woods & Plessinger, 1990). Researchers found that pregnant ewes showed enhanced effects of cocaine compared to nonpregnant ewes for variables like cocaine blood levels and heart rate. These findings suggest that cardiovascular and metabolic impacts of cocaine may be intensified during pregnancy further increasing risk of cocaine use at that time period. Despite these health concerns, little is known regarding how addiction to cocaine develops and progresses in the pregnant mother. It is likely that research related to addiction in pregnant mothers is limited because of the expansive literature detailing

health risks for the fetus and social stigma against the idea of addiction as a disease, especially during pregnancy.

Given these risks, there is a strong need to investigate current treatment options for women using cocaine and suffering from addiction during pregnancy. In terms of pharmacological treatments, there are currently no approved medications to treat cocaine use disorder in any population. Various medications can be prescribed to treat cocaine withdrawal symptoms, however these medications are not recommended for use during pregnancy due to potential adverse consequences for the developing fetus. For example, beta-blockers, like propranolol, are a particular class of drugs that have been used to reduce hypertension and anxiety associated with cocaine withdrawal (Kampman, 2005). With regards to pregnancy, one retrospective study found beta-blocker use during pregnancy to be associated with low birth weight and perinatal mortality (Meidahl Petersen et al., 2012). However, the aforementioned cocaine withdrawal symptom of hypertension may also have negative effects for the fetus depending on the gestational time period given that high systolic and diastolic blood pressure between eighteen weeks and thirty-six weeks gestation have been found to be associated with lower birth weight (Macdonald-Wallis et al., 2014). Unfortunately, research on mitigating this issue through studying the effect of maternal prescription drug use on fetal development is limited in data and primarily consists of case studies and retrospective, cohort studies. This is due to the ethical difficulty in experimentally treating pregnant women with such medications and measuring outcomes such as congenital malformations. Instead, psychosocial intervention may be more advantageous in pregnant women dealing with addiction. Pregnancy substance abuse programs have shown promise in improving health outcomes. In these specialized treatment programs, women undergo detoxification, participate in group counseling sessions, and

attend parenting classes (Weisdorf et al., 1999). Supplementing traditional approaches, consisting of lectures, group therapy sessions, and meditation, with pregnancy-focused education is more effective given that a significantly larger number of women who underwent the pregnancy substance abuse program completed their outpatient treatment compared to those who received traditional therapy. By connecting with other pregnant women facing similar issues and receiving instruction on how to care for the baby, women are more motivated to continue to accept help in eliminating substance use during pregnancy. Unfortunately, this treatment may not be as accessible as it could be given the barriers that exist and prevent pregnant women from seeking assistance in the first place. A qualitative study done by researchers at the Center for Addiction and Pregnancy revealed that women are hesitant to seek treatment during this vulnerable time for reasons that include hesitation due to stigma, limited information on how to join a program, and concerns about leaving a partner at home (Frazer et al., 2019). Women are also less likely to look for help given the complicated laws surrounding substance use and pregnancy. Nearly half the states in the United States consider substance use during pregnancy to be a form of child abuse which deters pregnant women even more from looking to receive treatment (*Substance Use During Pregnancy*, 2016). Further research must investigate safe pharmacotherapy options for pregnant women as well as how to combat the barriers that reduce their access to care.

Moreover, an understanding of the powerful nature of cocaine is necessary to conceptualize how these properties translate into addictive behaviors. Cocaine is a highly addictive drug that can enter the body in various ways including by smoking, injecting, or inhaling the drug. The bioavailability of cocaine varies as a function of the route of administration, specifically impacting how long it takes for cocaine to get into the bloodstream and reach target tissues. When considering cocaine plasma concentrations, a study done by Cone

(1995) compared the pharmacokinetics of intravenous, intranasal, and smoked routes of administration of cocaine. Peak plasma concentrations of 97.7-394.4 ng/ml and 154.0-354.1 ng/ml for intravenous and smoked routes of administration respectively, occurred at five minutes (Cone, 1995). In comparison, peak plasma concentrations rose slowly for intranasal administration to 40.1-88.5 ng/ml between twenty-three to fifty-one minutes. Self reports of subjective high experiences have also provided insight into the pharmacokinetics of various routes of cocaine use and may explain why people choose one method over another. Researchers utilized self reports from current cocaine abusers and found that participants reported a greater subjective high when intaking cocaine through a smoked versus inhaled route of administration (Volkow et al., 2000). In this same study, the time at which participants self-reported peak subjective highs was significantly faster for smoked compared to intravenous routes, and intravenous and smoked routes were significantly faster than intranasal administration of cocaine. Positron emission tomography (PET) scans identifying radioactively labeled cocaine have been used to assess the temporal activity of cocaine in the brain at the dopamine transporter (DAT) acting as a cocaine receptor (see below for additional discussion of the pharmacodynamics of cocaine). Although not studied for intranasal or smoked cocaine, for intravenous administration of cocaine, there is a strong relationship between the time course for cocaine uptake into the brain and the time course of subjective effects (Volkow et al., 1997). Therefore, given the observed differences in subjective highs, it would imply that those are driven by differences in the uptake of cocaine into the brain. To eliminate cocaine from the plasma, cocaine is hydrolyzed into the biologically inactive metabolites ecgonine methyl ester and benzoic acid by the enzyme butyrylcholinesterase (BChE; the primary enzyme involved in cocaine metabolism) (Schindler & Goldberg, 2012). Variability in this enzyme may be important

given that preliminary research has identified a relationship between BChE levels and cocaine use where, in one study, plasma levels showed higher BChE activity in cocaine addicts compared to controls (Munir et al., 2019).

The psychoactive effects of cocaine involve actions within the mesolimbic pathway that connect brain structures involved in motivation, reward, pleasure, and associative learning (Baik, 2013). The cell bodies in the ventral tegmental area (VTA) project to the nucleus accumbens and prefrontal cortex where the neurotransmitter dopamine is released (Baik, 2013). Studies have supported the idea that increases in brain dopamine levels are necessary for the rewarding effects of drugs of abuse. In one PET scan study, human participants that self-administered cocaine were found to have higher synaptic dopamine levels compared to placebo-treated participants as indicated by the decreased binding potential of a radioligand to dopamine receptors (Cox et al., 2009). This radioligand binding potential decreases as extracellular dopamine increases and occupies more of the dopamine receptors to outcompete the radioligand. Within this same study, participants treated with cocaine self-reported behavioral effects of cocaine such as higher energy and drug craving. The mechanism whereby cocaine may increase dopamine's availability in the synapse is via binding to the DAT which would normally be responsible for reuptake of dopamine from the synapse (*How Does Cocaine Produce Its Effects?*, 2016). Within the brain, these transporters are found at the highest levels in the striatum which includes the caudate nucleus, putamen, and nucleus accumbens. DATs are primarily localized to the axonal membranes of cells in the striatum linking their function to the mesolimbic pathway (Piccini, 2003). As previously mentioned, research using PET scans shows that DAT occupancy is positively correlated with the experience of being high, where increased blockage of DATs is associated with an increased subjective high within intravenous administration of cocaine

(Volkow et al., 1997). In order for any effects of cocaine to be felt, 47% of DATs had to be occupied where a dose of 0.3-0.6 mg/kg cocaine resulted in 60-77% of DATs being occupied. In addition to blocking DATs, studies have shown that cocaine also increases release of dopamine via synapsin-dependent reserve pools given that synapsin knockout mice exhibited reduced dopamine release following cocaine injections compared to wild-type controls (Venton et al., 2006). These data help to explain the mechanism of action through which cocaine increases dopamine levels to elicit the observed behavioral effects of reinforcement and reward.

Within the mesolimbic system, dopamine acts primarily on dopamine D1 and D2 receptors on medium spiny neurons (MSNs) in brain areas such as the nucleus accumbens core to mediate the effects of cocaine (Scofield et al., 2016). These dopamine D1 and D2 receptors on MSNs are G-protein coupled receptors that initiate intracellular signaling and typically exert opposite effects following dopamine binding (Scofield et al., 2016). D1 receptors specifically may play a role in drug reinforcement given that in one study, D1 receptor knock-out mice displayed significantly less acquisition of cocaine self-administration compared to wild-type mice (Caine et al., 2007). Additionally, the D1 knock-out mice did not show a difference in the self-administration of cocaine versus saline suggesting that activation of the D1 receptor specifically may be necessary for the reinforcement of cocaine. Together, these results indicate that increased dopamine release occurs during cocaine use and activation of dopamine receptors has implications in the rewarding effects of these drugs.

Researchers have begun to link addiction with hormone levels given that sex differences exist between males and females in terms of responses to cocaine, where women suffer from addiction and relapse at higher rates (Kokane & Perrotti, 2020). Women are subject to fluctuating levels of sex steroid hormones during their ovulatory cycle with progesterone and estradiol being

the main hormones involved. During pregnancy, concentrations of sex steroid hormones, specifically estradiol, are higher than at any other point of a female's reproductive cycle (Kumar & Magon, 2012). Serum estradiol levels continue to increase throughout the progression of a pregnancy as evidenced by one study that measured estradiol levels in pregnant women to be 0.87, 4.24, and 6.18 ng/ml at the first, second, and third trimester respectively (Soldin et al., 2005). Estradiol (produced primarily in the ovaries) elicits effects through activation of estrogen receptors in target tissues (Kumar & Magon, 2012). The two main estrogen receptors (ERs) found in the brain are ER-alpha and ER-beta (Weiser et al., 2008). In a classical pathway, estradiol and progesterone bind to intracellular receptors in the brain that dimerize upon activation (Kumar & Magon, 2012). The receptors then translocate to the nucleus and act as transcription factors by binding to hormone response elements and exerting their effects by modifying the transcription of deoxyribonucleic acid (DNA) (Kumar & Magon, 2012). Estradiol can also signal in a variety of nonclassical pathways, including via ERs that are localized in the plasma membrane (mERs). These mERs couple to membrane metabotropic glutamate receptors (mGluRs). Upon binding, a signaling cascade is initiated for fast-acting hormonal effects (Grove-Strawser et al., 2010). There is also diversity in the effects of ER activation in the brain, with much of the literature deriving from studies on neuroprotective effects and reproductive behaviors. For instance, researchers have found that estradiol increases the expression of genes that promote cell survival such as the B cell lymphoma 2 gene (Gollapudi & Oblinger, 1999). Studies with rodent models have also demonstrated evidence of ER activation in the brain leading to female sexual behavior including proceptive (solicitational) and receptive (lordosis) responses. For example, it was found that reducing the expression of ER-alpha in the

ventromedial hypothalamus blocked the upregulation of progesterone receptor expression and inhibited sexual behavior in ovariectomized female rats (Musatov et al., 2006).

The mesolimbic pathway of reward and reinforcement associated with addiction and cocaine use is sensitive to these aforementioned fluctuating levels of female sex steroid hormones (Kokane & Perrotti, 2020). In one study, female rats were assessed for the reinstatement of cocaine-seeking behavior through lever pressing (Feltenstein et al., 2011). There was a significantly higher incidence of reinstatement for rats in the proestrus phase of their cycle, the time at which estradiol levels are highest. Similarly, female rats that were ovariectomized and treated with estradiol showed increased lever pressing for self-administration of intravenous cocaine compared to ovariectomized females treated with vehicle (Lynch et al., 2001). Though data supports this effect, the mechanism of action that may underlie estrogen's influence on cocaine-induced responses in females is not well understood. The nonclassical membrane estrogen signaling pathway is one area of interest in attempting to explain this phenomenon. Estradiol has been found to act on MSNs in the neostriatum and elicit fast-acting effects as shown in one study where researchers found that treating neostriatal tissue from female rats with estradiol resulted in a reduction in Ba^{2+} currents through Ca^{2+} channels (Mermelstein et al., 1996). Of particular note, activation of mER-alpha specifically was found to trigger a rapid response, within the order of minutes, as indicated by increased cAMP-responsive element binding (CREB) phosphorylation (Grove-Strawser et al., 2010). These rapid effects of estradiol in the brain may be due in part to the fact that mER-alpha is coupled to mGluR5, which is found in the MSNs of the nucleus accumbens (Tonn Eisinger et al., 2018). This mechanism has been investigated by assessing dendritic spine plasticity. Prior studies have looked at how treatment with cocaine causes dendritic spine plasticity and may underlie the behavioral changes associated

with cocaine use (Robinson & Kolb, 2004). Similarly, estradiol has been shown to decrease dendritic spine density in the NAc core (Staffend et al., 2011). When thinking about a mechanism whereby this may occur, animals treated with estradiol and an mGluR5 antagonist did not display the expected reduction in dendritic spine density of MSNs suggesting that activation of mGluR5 is involved in this estradiol-mediated pathway (Peterson et al., 2015). This has also been supported through behavioral experiments where females treated with an mGluR5 antagonist showed significantly less cocaine self-administration compared to those treated with vehicle (Martinez et al., 2016). Given these changes in both dendritic spine plasticity and responses to cocaine, estradiol may enhance behavioral responses to cocaine through activation of mER-alpha coupled to mGluR5 in MSNs of the nucleus accumbens.

Another related explanation may be due to the impact of estradiol on dopamine release. A study done by Yoest et al. (2019) explored how estradiol may impact cocaine-induced dopamine release. By utilizing fast-scan voltammetry, researchers compared dopamine release in the NAc shell following cocaine treatment in animals treated with estradiol or vehicle (Yoest et al., 2019). They found that animals treated with estradiol displayed higher levels of cocaine-induced dopamine release. The mechanism whereby estradiol interacts with dopamine to elicit such effects is not well understood, though researchers have proposed various hypotheses. Of particular note, differences in binding at dopamine receptors may underlie these effects. In one study, researchers compared dopamine D2 receptor specific binding in the NAc core and found that ovariectomized rats treated with estradiol displayed increased D2 receptor binding compared to rats treated with vehicle (Le Saux et al., 2006). Interestingly, animals treated with an ER-beta agonist also displayed this increase in D2 receptor specific binding. Given dopamine's involvement in the reinforcing and rewarding properties of cocaine, these data suggest that

estradiol may elicit enhanced responses to cocaine through increasing dopamine D2 receptor binding.

As discussed, in recent years estradiol has become a target for researchers to attempt to explain motivation for cocaine within animal models of drug addiction. The current study builds off a study done by Segarra et al. (2014) on gonadally intact, female nonpregnant rats that were treated with the estrogen receptor antagonist 7α,17β-[9-[(4,4,5,5,5-Pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol (ICI) through intracerebroventricular (ICV) administration. These animals underwent behavioral conditioned place preference (CPP) testing where they were conditioned with cocaine in a two-chamber apparatus to assess the extent to which they were motivated to seek cocaine (Segarra et al., 2014). They found that treatment with ICI disrupted the formation of a CPP for cocaine. Based on these results, failure to form a CPP for cocaine would indicate that estradiol signaling is involved in the animals' motivation for cocaine and contributes to the reinforcing properties of cocaine. Further studies have looked at targeting specific estrogen receptors as well as brain areas that may regulate the rewarding properties of cocaine in rodent models. One study found that a knockdown of ER-beta in the nucleus accumbens diminished a CPP for cocaine in female mice (Satta et al., 2018). This study also found that ovariectomized mice treated with estradiol or an ER-beta agonist displayed an increased CPP for cocaine compared to vehicle animals. When considering the impact of hormone levels associated with pregnancy, one study looked at motivation for cocaine during various stages of the reproductive process in female rats (Hecht et al., 1999). Motivation for cocaine was assessed through progressive ratio self-administration of intravenous cocaine where animals were required to increase their number of responses in order for the drug to be delivered. Researchers found that female rats displayed

the highest number of lever presses during the estrus period and first trimester of pregnancy, with the lowest levels occurring near the time of birth. Despite the dramatic increase in estradiol that occurs across the first two trimesters of pregnancy, there does not appear to be a significant difference in motivation for cocaine compared to nonpregnant animals in the estrus phase of their reproductive cycle. This idea that high estradiol levels do not necessarily relate to increased behavioral responding has been shown in studies on maternal behavior. Researchers have also found that female rats in their 15-17th day of pregnancy displayed a median three day latency prior to the onset of maternal behaviors, like pup retrieval, when exposed to pups within their cage (Rosenblatt & Siegel, 1975). Similarly, after day three of exposure to pups, there were increases in maternal behaviors in nonpregnant animals which shows very comparable latencies to pregnant animals in maternal behavior. These data suggest that increasing brain estradiol levels to pregnancy levels does not necessarily cause enhanced behavioral responses for behaviors that are sensitive to estradiol.

Research has not yet integrated pregnancy and hormonal manipulation to understand how pregnancy and associated increased levels of estradiol during this time period may factor into motivation for cocaine. Extending this research towards a pregnant population is important given the aforementioned risks and limited treatment options associated with cocaine addiction during pregnancy. In the current study, the goal is to investigate the role of estradiol signaling in motivation for cocaine during pregnancy. We hypothesized that estradiol acts at estrogen receptors in brain areas important for motivation to facilitate behavioral responses to cocaine in pregnant rats. Given that estrus females and females in mid-pregnancy display comparable progressive ratio responses for the self-administration of cocaine, we expected that pregnant animals would form a CPP for cocaine that is comparable to nonpregnant animals (Hecht et al.,

1999). In addition, we predicted that ICV administration of the estrogen receptor antagonist ICI in pregnant animals would disrupt the formation of a CPP for cocaine given that ICI disrupts the formation of a CPP for cocaine in nonpregnant animals (Segarra et al., 2014).

Materials and Methods

Overview of Experimental Design

In experiment 1, the goal was to determine if pregnant rats formed a conditioned place preference (CPP) for cocaine at a comparable level to nonpregnant rats (Figure 1A). In this experiment, pregnant and nonpregnant female rats were assessed by their motivation for 10 mg/kg cocaine using a biased-design CPP protocol.

In experiment 2, the role of signaling at estradiol receptors in motivation for cocaine was assessed in pregnant females. Specifically, pregnant animals underwent stereotaxic surgery to receive an implant providing continuous intracerebroventricular delivery of the estrogen receptor antagonist ICI or vehicle. Animals were then assessed by their motivation for cocaine through CPP testing. The CPP protocol was either identical to experiment 1 (experiment 2a; Figure 1B), or modified to incorporate a secondary pretest to address issues with chamber preference reliability in pregnant females (experiment 2b; Figure 1C). At the conclusion of behavioral testing, animal brains were collected, sectioned, and stained for histological analysis of the location of termination for the cannula implant.

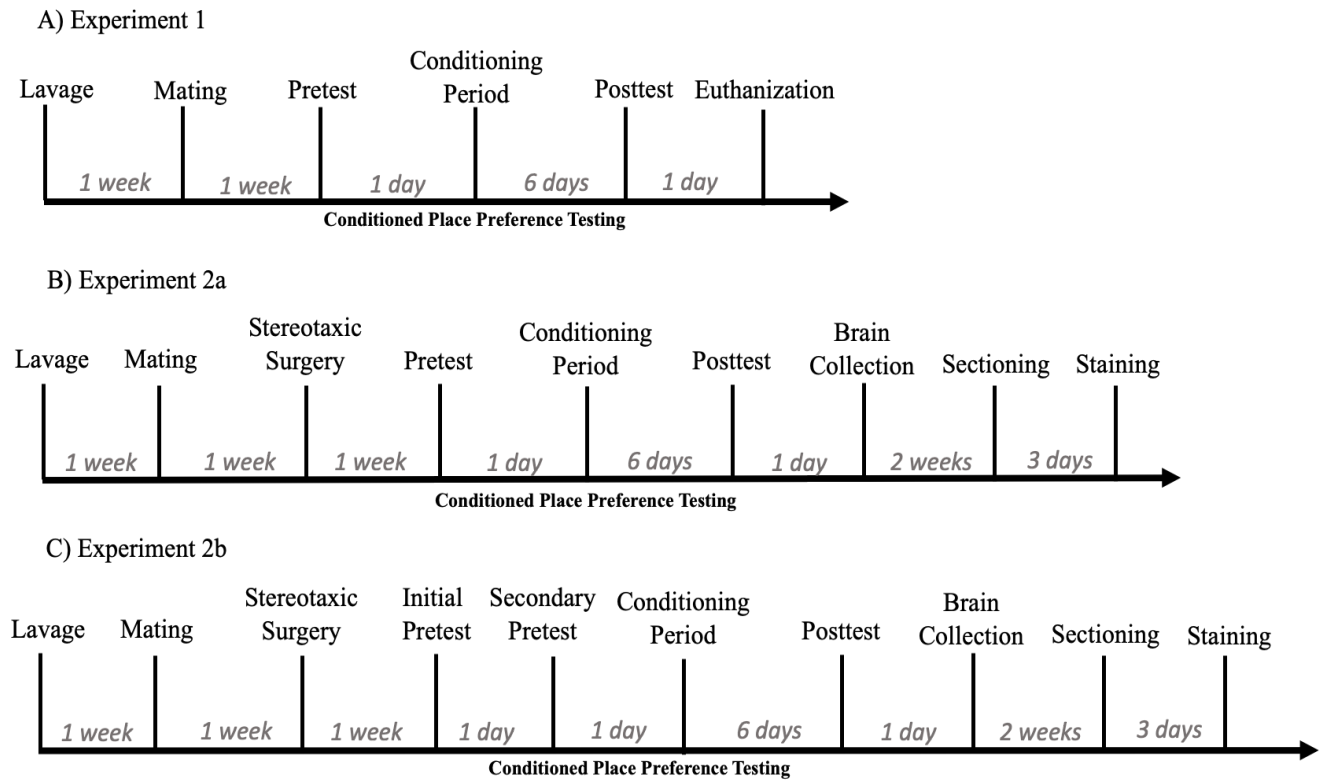

Figure 1. Timelines of experimental design

Animals

Female Sprague-Dawley rats were offspring of first generation breeder animals that had originally been obtained from the Charles River Laboratory (Kingston, NY). Animals were pair-housed in wire-top polycarbonate cages following weaning at 3 weeks of age and maintained on a 12:12 light-dark cycle with lights shutting off at 12:00 pm. Animals were between 10 and 12 weeks of age at the initiation of the experiment, which is typically viewed as young adulthood (Sengupta, 2013). All animals had *ad libitum* access to food (LabDiet 5001; F. Fisher & Son, Somerville, NJ, USA) and water. Animal procedures were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* and approved by the Trinity College Animal Care and Use Committee.

Drugs and Injections

Cocaine hydrochloride (C5776; Sigma-Aldrich, St. Louis, MO, USA) dissolved in sterile saline (10 mg/ml) was injected intraperitoneally at a dose of 10 mg/kg. Animals were given injection volumes of 1 ml/kg of body weight. This dose has previously been found as sufficient for the formation of a CPP in female rats (Zakharova et al., 2009). The estrogen receptor antagonist ICI (1047; Tocris Bioscience, Pittsburg, PA, USA) with a concentration of 0.5 mg/ml at a dose of 1.8 μg/day or vehicle was delivered intracerebroventricularly through a cannula implant pump (2006; Alzet, Cupertino, CA, USA) following surgery, as previously described by Segarra et al. (2014). Vehicle solutions were prepared with 50% dimethyl sulfoxide (D12345; Thermo Scientific, Waltham, MA, USA), 35% water, and 15% ethanol.

Experiment 1

Lavage and Mating

All animals in the pregnant treatment group underwent a lavage and mating protocol to ensure that they would each be successfully impregnated and go through the rest of the methodology at the same stage of their pregnancy. Nonpregnant animals were not included in this portion of the methodology. The lavage sampling technique was utilized after lights went out to determine the stage of the rat's estrous cycle and therefore when females would be sexually receptive. Animals were gently restrained in an upside-down orientation while approximately 100 μl of saline was flushed into the vaginal canal of the rats using a 1000 μl micropipette set to 150 μl. The saline was infused 3-4 times before recollecting the liquid and pipetting onto a slide. Uncoverslipped slides were viewed wet under an OMAX (M82ES-SC100-LP100) light microscope at 10x magnification, and ToupView software allowed for the visualization of cells in the samples using photomicrographs. If females were determined to be in proestrus,

characterized by clumps of ovoid, nucleated round cells in addition to the absence of leukocytes, they were paired with a sexually-experienced male rat. In order to be mated overnight, female rats had to show proceptive (i.e., ear wiggling) and/or receptive (i.e., lordosis) responses to the male. The day of mating became day 0 of pregnancy. Animals were weighed daily from day 0 of pregnancy through the end of the experiment.

Conditioned Place Preference Testing

All animals underwent conditioned place preference (CPP) testing to assess their ability to associate stimuli from a particular chamber in a two-chamber apparatus (61 x 30 x 30 (l x w x h) cm) with the reinforcing effects of cocaine. Differing stimuli came in the form of various visual and tactile information where one chamber of the apparatus had striped walls and square flooring whereas the other chamber had checkered walls and circular flooring. Removable doors separated the two chambers. Light intensity was measured at the center and all corners of both chambers. Light intensity was maintained at 2.7 lux or less with a deviation of up to 0.1 lux for mirrored locations between the two chambers, in order to prevent any influence of light intensity differences within the chambers on time spent in either chamber.

Animals began behavioral testing on day 7 of their pregnancy for pregnant animals. Beginning with a pretest, the doors separating the two chambers were removed, and animals were allowed to move freely between the two chambers of the apparatus for fifteen minutes. These pretest sessions were recorded which allowed for the assessment of their time spent in each chamber through JWatcher software where scorers designated keys for the left and right chambers (Blumstein et al., 2020). The appropriate keys were clicked once the front of the hind leg had crossed over to a particular chamber. Distance traveled data were collected through either IDTracker (Perez-Escuerdo et al., 2014) software or ezTrack (Pennington et al., 2019) software,

which identified the animal's center of mass in the chamber at each frame of the video to calculate the linear discrepancy in center mass location between frames. Following the scoring of the pretest, an initially preferred and non-preferred chamber was determined for each animal. Animals then went through a six-day conditioning period where animals randomly assigned to the cocaine treatment group received intraperitoneal injections of 10 mg/kg cocaine before being placed in their initially nonpreferred chamber on days 1, 3, and 5. On days 2, 4, and 6, animals received saline in their initially preferred chamber. Animals spent 30 minutes per day in the appropriate chamber immediately following their injection. Animals assigned to the saline treatment group received saline injections for each day of conditioning. During these conditioning sessions, the doors between the chambers remained closed. Following conditioning, animals underwent a posttest with an identical set up to the pretest where time spent in each chamber was scored and distance traveled was measured. CPP scores were calculated by measuring the difference in the amount of time spent in the initially non-preferred chamber during the posttest compared to the pretest. All animals were euthanized the day following their posttest.

Experiment 2

Lavage and Mating

Lavage and mating protocol was identical to experiment 1 given that all animals in experiment 2 were part of a pregnant treatment group.

Stereotaxic Surgery

All animals underwent a stereotaxic procedure to implant an intracerebroventricular (ICV) cannula connected to a pump for drug delivery to the target location. On day 0 of pregnancy, a mini osmotic pump was prepared for both ICI and vehicle drugs. A vinyl catheter

tube was cut to 10 cm and attached to the cannula on one end and to the flow modulator on the other. The pump was then weighed to measure the empty weight prior to adding the drug. A 1 ml syringe filled to 0.5 ml of vehicle or ICI was used to fill the pump until liquid could be seen at the opening. The pump was then reweighed to measure the filled weight and ensure that the pump contained at least its mean fill volume of 223.8 µl. Pumps were attached to the flow modulators and delivered the drug at a rate of 0.15 µl per hour. Once the pump was assembled, it remained in a saline-filled 15 ml conical tube covered with aluminum foil overnight at 37 degrees Celsius for incubation.

On day 1 of pregnancy, animals underwent stereotaxic surgery to implant the pump with either ICI or vehicle for drug delivery to the left lateral ventricle of the brain. The anesthetic isoflurane (14043-704-06; Patterson Veterinary, Greeley, CO, USA) was loaded into a 10 ml glass syringe and primed in the SomnoSuite Small Animal Anesthesia System (SS6346B; Kent Scientific, Torrington, CT, USA) which administers anesthesia through the course of the surgery. First, animals were placed in an induction box receiving isoflurane dissolved in oxygen gas at a concentration of 5% v/v to induce the anesthetic state. Once breathing rate slowed to approximately one breath per second, animals were switched to a nose cone administering 2-3.5% isoflurane to maintain the anesthetic state. Animals received a subcutaneous injection of 2.5 mg/kg meloxicam (137590E; Pivetal, Liberty, MO, USA) diluted in sterile saline for analgesic purposes. Animals were given injection volumes of 1 ml/kg of body weight. Fur from the nape of the neck and on the scalp was shaved and eye ointment (701039; Major Pharmaceuticals, Livonia, MI, USA) was swabbed onto both eyes of the animals to lubricate the eyes through the course of the surgery. The shaved portion of the animals was then wiped with 70% ethanol and betadine (67618-154-16; Aviro Health, Stamford, CT, USA) twice to prevent

infection and sterilize the surgical site. Animals were transferred to the stereotaxic apparatus (51603U; Stoelting, Wood Dale, IL, USA) and the front teeth were latched onto the incisor bar that was incorporated into the anesthesia mask for continued delivery of anesthesia. A heating pad worked to maintain animal body temperature, while a rectal thermometer was inserted to measure the temperature. Ear bars were positioned anterior to the ear canals of the animals to keep the head of the animal level and immovable relative to the apparatus during the procedure. Using a single blade, a 3 cm incision down the top of the head was made and connective tissue was cleared using the wooden end of cotton swabs. A stereotaxic arm with an attached cannula holder was secured to the apparatus, and then the cannula was secured into the cannula holder. Several drops of a hydrogen peroxide solution (H325-500; Fisher Scientific, Fair Lawn, NJ, USA) onto the skull accentuated the skull line intersections of interest where cranial bones were fused together, notably lambda and bregma. Bregma was identified where the frontal bone and two parietal bones fused together, while lambda was where the two parietal bones fused to the occipital bone. By using the tip of the cannula, the skull was leveled to where lambda and bregma were within 0.05 mm of each other on a dorsal/ventral plane. The cannula was then moved to +1.70 mm lateral and -0.50 mm posterior relative to bregma and marked with a pencil at this position, and a hole was drilled down at the marked location using a foot-pedal operated drill (G170773; Foredom, Bethel, CT, USA). An additional two holes to the right of the cannula entry hole were drilled for placement of the screws. One hole was drilled 1 mm posterior relative to bregma and 3 mm lateral to the right of the midline, and the second hole was drilled posterior and 3 mm from the other screw hole. For the additional two holes, 3.2 mm long jeweler screws with a 1.59 mm outer diameter were screwed into the holes using a pocket clip slotted screwdriver. A 0.5 mm spacer (2006; Alzet, Cupertino, CA, USA) was placed above the cannula

entry hole in order to ensure that the tip of the cannula terminated within our target ventricle. The cannula was then dropped down through the spacer hole, past the skull, and into the left lateral ventricle of the brain.

Cyanoacrylate glue was used to initially secure the cannula to the skull and connect the screws with the skull and cannula. The stereotaxic arm was then detached leaving behind the cannula with the tubing attached to the pump. A cement cap was assembled on top of the skull reaching up to the base of the cannula using mixed dental cement powder (51458; Stoelting, Wood Dale, IL, USA) and liquid (51456; Stoelting, Wood Dale, IL, USA). A four centimeter incision of the skin down the neck was made to allow for placement of the pump subcutaneously adjacent to the thoracic spine. Nine millimeter stainless steel wound clips (NC0185806; Fisher Scientific, Waltham, MA, USA) were used to close the skin incision. Triple antibiotic ointment (69396-002-20; Globe, Fort Lauderdale, FL, USA) was administered down the incision on top of the wound clips to prevent infection at the incision site. At the conclusion of the surgery, animals were removed from the stereotaxic apparatus and returned to their cage for postoperative monitoring.

Following surgery, animals received a 5 mg/ml carprofen or meloxicam subcutaneous injection for pain relief on postoperative day 1 and were assessed for brightness, alertness, and responsiveness (BAR) throughout the three day postoperative monitoring period. Animals either remained single-housed for the duration of the experiment (experiment 2a) or were pair-housed after a one-week surgical recovery period (experiment 2b).

Conditioned Place Preference Testing

Animals began behavioral testing following a one-week surgical recovery period. In experiment 2a, animals underwent CPP testing identical to experiment 1. In experiment 2b, the

CPP testing protocol was modified to incorporate a secondary pretest 24 hours following the initial pretest. This secondary pretest was then used for establishing initial chamber preferences and calculating CPP scores.

Brain Sectioning and Cresyl Violet Staining

The day after the posttest, animals were visually assessed to confirm pregnancy through the swelling of the abdomen and the appearance of nipples on the underside. Animals were then euthanized using isoflurane in a glass chamber. Secondary confirmation of euthanization occurred through cervical dislocation.

Animals were then decapitated and their brains were removed and fixed with 10% formalin (SF100-20; Fisher Scientific, Baltimore, MD, USA) in a glass vial for storage for up to two weeks. Brain removal allowed for imaging of damage to the cortex and successful or unsuccessful entry of the tip of the cannula into the left lateral ventricle. In order to be imaged, brains were first sectioned using a Vibratome (074018; Vibratome, St. Louis, MO, USA). Brains were deposited from the vial and excess moisture was removed from the surface of the tissue to allow for horizontal cutting. A razor blade was used to cut the brain -5.5 mm relative to bregma with the ventral side of the brain facing upwards. Cut brains were glued using cyanoacrylate glue to a metal disc that was securely mounted to the Vibratome. After allowing 10 minutes for drying of the glue, the inner well of the Vibratome was filled with phosphate-buffered saline solution and the outer well was filled with ice. Phosphate-buffered saline was prepared with 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, 800 ml deionized water, and water added to make a final volume of 1 liter of solution. Once a blade was inserted into the Vibratome, brains were sectioned at 250 micrometer thin slices with 50 micrometer increment decreases down to 50 micrometer slices as neuroanatomical references suggested that sectioning was nearing the

beginning of the damage. Points of reference for when to begin mounting brain slices were indicated using The Rat Brain in Stereotaxic Coordinates atlas (Paxinos & Watson, 2009) and included when the anterior commissures began to come together, when the corpus callosum had fully come together, and when the lateral ventricles began to appear. Superfrost plus microscope slides (1255015; Fisher Scientific, Pittsburg, PA, USA) for mounting were dipped in 37 degrees Celsius gelatin solution (G9391; Sigma-Aldrich, St. Louis, MO, USA), and brain slices were mounted onto the slides when the damage began to appear. Damage from the cannula appeared to enter through the cortex in a thin, straight line through the corpus callosum and into the left lateral ventricle. Mounting of slices continued until damage through the corpus callosum was no longer evident.

Following sectioning, slides were stained using a cresyl violet staining procedure (Deyo, 2013) as outlined in Table 1. This staining technique identified the soma of neurons in the brain tissue as a violet color which allowed the tissue to be imaged with precise identification of the location of the cannula entrance. Cresyl violet solution was prepared with 282 ml 0.1 M acetic acid, 18 ml 0.1 M sodium acetate, and 30 ml cresyl violet stock solution (J64318-09; Thermo Scientific, Waltham, MA, USA). Stained slides were coverslipped using DPX mounting medium (13512; Electron Microscopy Sciences, Hatfield, PA, USA). After a 24 hour period for drying, stained slides were then viewed under a microscope (SZ40; Olympus) using Toupview software. If the position of tip of the cannula was determined to be outside of the ventricle, suggesting that the animal did not receive the drug in the target location, data collected for the animal was excluded from the experiment.

Step	Solution	Duration (min)
1	CitriSolv (1601; Decon Labs, King of Prussia, PA, USA)	5
2	95% ethanol	3
3	70% ethanol	3
4	Deionized water	3
5	Cresyl violet at 60 degrees Celsius	11
6	Deionized water	3
7	70% ethanol	3
8	95% ethanol	1
9	100% ethanol	1 dip
10	CitriSolv	5
11	CitriSolv	30

Table 1. Cresyl Violet Staining Procedure

Data Analysis

All data were statistically analyzed using JASP software for Macintosh, version 0.17.1 (JASP Team, 2023). For experiment 1, data were analyzed for the effects of pregnancy status (pregnant or nonpregnant) and drug treatment (0 mg/kg or 10 mg/kg cocaine) on CPP score and

percent weight change using factorial ANOVAs. Time (pretest or posttest), pregnancy status, and drug treatment were all tested for their effects on the average distance traveled using repeated measures ANOVAs. Significant interactions were further analyzed through simple main effects to look at the effect of time by drug treatment on average distance traveled. One way ANOVAs were run for the effect of drug treatment and pregnancy status on average distance traveled for the pretest and posttest. To do so, separate factorial ANOVAs were run examining the effects of drug treatment and pregnancy status at each testing time point.For experiment 2, the effects of hormone condition (ICI or vehicle) and drug treatment on CPP score were examined using factorial ANOVAs. Data from this experiment were also analyzed for the effects of time, hormone condition and drug treatment on mean distance traveled and the change in weight from baseline using repeated measures ANOVAs. A significant interaction between hormone condition and drug treatment on the average distance traveled was followed up by running an ANCOVA with pretest distance traveled as a covariate. In all cases, results were considered statistically significant if the p value was less than 0.05.

Results

Experiment 1

These data were analyzed using a factorial ANOVA to assess if pregnant animals found a dose of 10 mg/kg cocaine rewarding to a similar degree as nonpregnant animals. It was found that there was no significant effect of pregnancy condition on CPP score, $F(1, 26) < .001$, $p = 0.979$ (Figure 2). There was a significant effect of drug treatment on CPP score with an increased score for animals treated with 10 mg/kg cocaine compared to 0 mg/kg cocaine, $F(1, 26) = 10.646$, $p = 0.003$. In order to confirm that pregnant females are able to successfully form a CPP for cocaine, these data were further examined for the simple main effect of drug treatment within

each level of pregnancy status. Pregnant animals treated with 10 mg/kg cocaine had a significantly higher CPP score than pregnant animals treated with 0 mg/kg cocaine $F(1,26) = 7.220$, $p = 0.012$, similar to what was observed in nonpregnant animals, $F(1,26) = 4.795$, $p = 0.038$. The magnitude of the CPP for cocaine was comparable across nonpregnant and pregnant animals as evidenced by a nonsignificant pregnancy status by drug treatment interaction, $F(1, 26) = 0.238$, $p = 0.630$.

Effect of Pregnancy on CPP Score

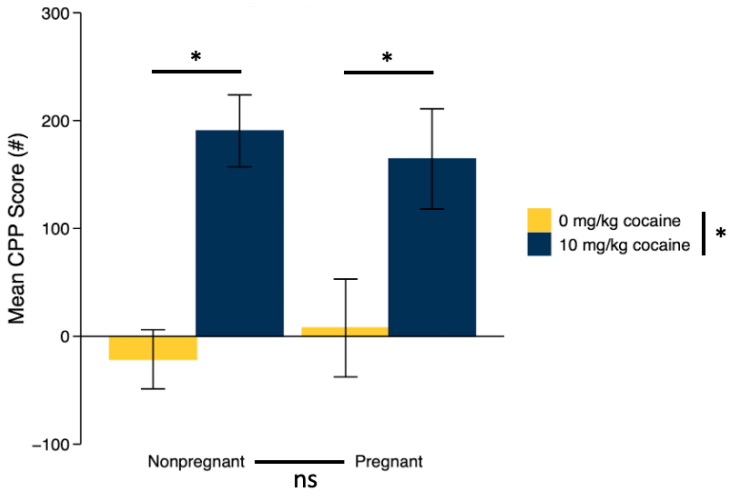

Figure 2. Mean (+/- SEM) conditioned place preference (CPP) scores for nonpregnant and pregnant animals conditioned with 0 mg/kg cocaine or 10 mg/kg cocaine. *$p < 0.05$ (main effect of drug treatment and simple main effect of drug treatment on CPP score at both levels of pregnancy status); ns, not significant (main effect of pregnancy status).

A factorial ANOVA looked at differences in weight progression for nonpregnant and pregnant animals based on drug treatment in order to confirm typical weight gain patterns in both groups. As shown in Figure 3, there was a significant effect of pregnancy condition on the mean

percent weight change from pretest to posttest with pregnant animals showing greater percent increase compared to nonpregnant animals, $F(1, 26) = 24.736$, $p < .001$. There was no significant effect of drug treatment on mean percent weight change, $F(1, 26) = 0.013$, $p = 0.909$. There was also no significant interaction between pregnancy condition and drug treatment on mean percent weight change, $F(1, 26) = 0.185$, $p = 0.671$. These data indicate that cocaine treatment did not significantly influence weight gain patterns in either group.

Effect of Pregnancy on Weight Increase

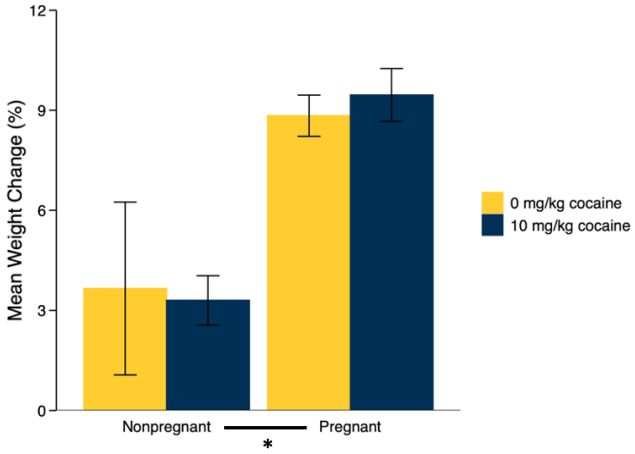

Figure 3. Mean (+/- SEM) percent weight change between the pretest and posttest for nonpregnant and pregnant animals conditioned with either 0 mg/kg cocaine or 10 mg/kg cocaine. *$p < 0.05$ (main effect of pregnancy status).

To investigate average distance traveled at the pretest and posttest depending on the drug treatment and pregnancy status of animals, a repeated measures ANOVA was run. Data from Figure 4 depicts that there was a significant main effect of time on average distance traveled with an increased distance traveled at the pretest compared to the posttest, $F(1, 26) = 24.212$, $p < .001$.

Nonpregnant and pregnant animals were found to travel similar distances given that there was no significant effect of pregnancy status on average distance traveled between pretest and posttest, $F(1, 26) = 1.629$, $p = 0.213$. There was also a significant interaction between time and drug treatment, $F(1, 26) = 7.247$, $p = 0.012$. This interaction was analyzed at the pretest and posttest for differences in distance traveled based on drug treatment. For the pretest, there was no significant effect of drug treatment on distance traveled, $F(1, 26) < .001$, $p = 0.987$. For the posttest, there was a significant effect of drug treatment on distance traveled where animals treated with 0 mg/kg cocaine traveled less than animals treated with 10 mg/kg cocaine, $F(1, 26) = 4.919$, $p < 0.036$. This interaction was further analyzed to show that animals treated with 0 mg/kg cocaine significantly decreased their average distance traveled between the pretest and posttest, which explains why the time by drug treatment interaction exists, $F(1, 26) = 30.646$, $p < .001$. In contrast, animals treated with 10 mg/kg cocaine showed no significant difference in their average distance traveled between the pretest and posttest, $F(1, 26) = 2.355$, $p = 0.149$.

Effect of Time and Drug Treatment on Distance Traveled

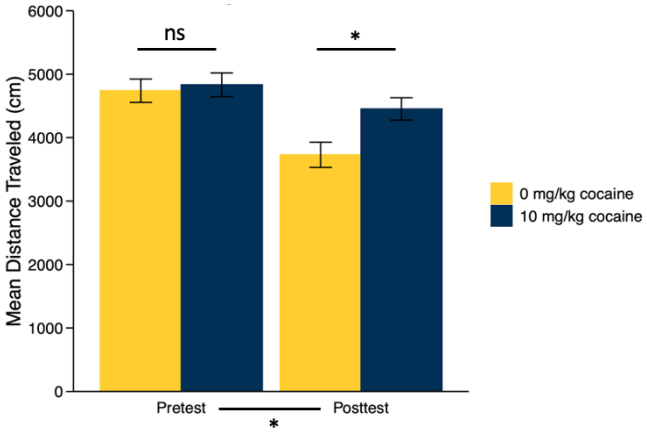

Figure 4. Mean (+/- SEM) distance traveled at the pretest and posttest for animals conditioned with 0 mg/kg cocaine or 10 mg/kg cocaine. Data were collapsed across pregnancy status. *p <

0.05 (main effect of time and simple main effect of drug treatment at posttest); ns, not significant (simple main effect of drug treatment at pretest).

Experiment 2

Given that pregnant animals formed a CPP for 10 mg/kg cocaine at a comparable value to nonpregnant animals, the role of hormone manipulation during pregnancy on the reinforcing properties of cocaine was explored. A factorial ANOVA was run to assess if blocking estradiol receptors through ICI impacted pregnant animals' motivation for cocaine as measured by a CPP for 10 mg/kg cocaine. Because there was no significant effect of hormone condition on CPP score, ICI treatment did not impact pregnant animals' motivation for 10 mg/kg cocaine, $F(1, 13) = 0.048$, $p = 0.830$ (Figure 5). Surprisingly, it was found that there was no significant effect of drug treatment on CPP score as shown by how animals treated with 0 mg/kg cocaine or 10 mg/kg had similar CPP scores, $F(1, 13) = 0.038$, $p = 0.848$. There was also no significant interaction of drug by hormone condition, $F(1, 13) = 0.020$, $p = 0.890$. The lack of any cocaine effect on CPP scores makes it difficult to intercept the effects of ICI on these scores.

Effect of Hormone Condition on CPP Score

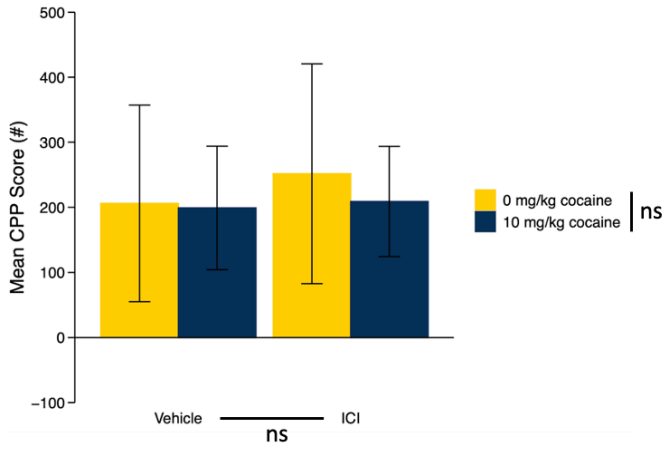

Figure 5. Mean (+/- SEM) conditioned place preference (CPP) scores for animals receiving hormone conditions of ICI or vehicle and conditioned with 0 mg/kg cocaine or 10 mg/kg cocaine. ns, not significant (main effects of hormone condition and drug treatment).

A repeated measures ANOVA investigated the effect of hormone condition and drug treatment on the change in weight from baseline at the pretest and posttest. As shown in Figure 6, there was a significant main effect of time on the change in weight from baseline with an expected increased change in weight at the posttest compared to the pretest, $F(1, 13) = 181.316$, $p < .001$. There was also a significant between subjects effect of hormone condition on the change in weight from baseline where animals treated with ICI displayed an increased change in weight compared to animals treated with vehicle, $F(1, 13) = 14.039$, $p = 0.002$.

Effect of Time and Hormone Condition on Weight Increase

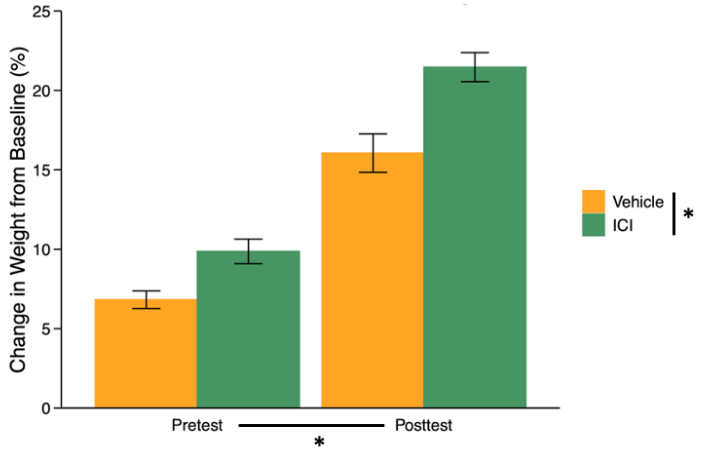

Figure 6. Mean (+/- SEM) percent weight change from baseline at the pretest and posttest for animals treated with vehicle or ICI. Data were collapsed across drug treatment. *$p < 0.05$ (main effect of time and hormone condition).

Animal distances traveled at the pretest and posttest were analyzed based on hormone condition and drug treatment through a repeated measures ANOVA. Animals traveled more during the posttest based on the significant main effect of time on average distance traveled, $F(1, 13) = 21.843$, $p < .001$. There was also a significant between subjects effect of drug treatment on the average distance traveled with an increased distance traveled for animals treated with 10 mg/kg compared to 0 mg/kg cocaine, $F(1, 13) = 8.059$, $p = 0.014$. Because animals were not injected with cocaine or saline at the pretest, a between subjects effect of drug treatment on distance traveled is not possible. To account for potential preexisting differences in treatment groups, an ANCOVA adjusted with the pretest distance traveled as a covariate was run. It was found that there is a significant interaction between hormone condition and drug treatment on the average posttest distance traveled, $F(1, 12) = 9.881$, $p = 0.008$. To follow up on this interaction, a simple main effect of hormone condition was run for the average posttest distance traveled at both levels of the drug treatment. At 0 mg/kg, there was a larger distance traveled for animals treated with vehicle compared to ICI, $F(1, 12) = 6.984$, $p = 0.021$ (Figure 7). Contrastingly, at 10 mg/kg, there was a larger distance traveled for animals treated with ICI compared to vehicle, $F(1, 12) = 5.986$, $p = 0.031$.

Effect of Hormone Condition and Drug Treatment on Distance Traveled

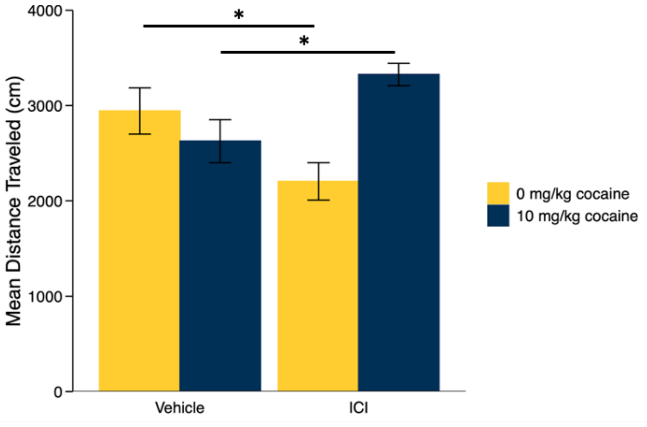

Figure 7. Mean (+/- SEM) distance traveled at the posttest for animals treated with vehicle or ICI and conditioned with 0 mg/kg cocaine or 10 mg/kg cocaine. Posttest distance traveled data were analyzed through an ANCOVA with pretest distance traveled data as a covariate. *$p < 0.05$ (simple main effect of hormone condition on mean distance traveled at 0 mg/kg and 10 mg/kg).

Discussion

Experiment 1 sought to identify whether pregnant animals formed a CPP for cocaine at a comparable level to nonpregnant animals. In a previous study, adult female rats formed a significant CPP for cocaine at a dose of 10 mg/kg (Zakharova et al., 2009). These results were also demonstrated in the current study where nonpregnant animals displayed motivation for cocaine through robust CPP scores for 10 mg/kg cocaine. Importantly, pregnant animals treated with 10 mg/kg cocaine formed a CPP that was significantly different from pregnant animals treated with saline only, but not different from nonpregnant animals treated with 10 mg/kg cocaine. These findings indicated that a dose of 10 mg/kg cocaine could be used to assess motivation for cocaine in pregnant animals as seen in our subsequent experiments. Experiment 2 looked at how blocking estrogen receptors using ICI impacted motivation for 10 mg/kg cocaine in pregnant animals. The results from this experiment were unexpected in that there was no difference in CPP score between pregnant animals treated with vehicle compared to animals treated with ICI. Additionally, there was no difference in CPP score for animals treated with 0 mg/kg cocaine or 10 mg/kg cocaine. It is important to note that pregnant animals treated with vehicle in experiment 2 were experimentally comparable to pregnant animals with no hormonal

manipulation in experiment 1. Yet, pregnant animals were not found to be motivated for 10 mg/kg cocaine when treated with vehicle.

Two possible explanations for the CPP results of the current study have been investigated through exploratory experimentation. First, single-housing of animals in experiment 2a was identified as a potential influence on CPP score. Single-housing was chosen in the methodology to avoid potential removal of wound clips if animals were to groom each other. It has previously been found that isolated female rats showed increased motivation for cocaine via self-administration compared to animals that were group-housed (Westenbroek et al., 2013). Therefore, motivation for cocaine may have been expected to influence CPP results where isolated pregnant animals would show increased CPP scores. However, this prior study does not support the lack of a CPP for cocaine in pregnant animals observed here in experiment 2a. Regardless, social housing was chosen for pregnant animals in experiment 2b as a modification in order to better mimic the setup that was successful in experiment 1.

Moreover, the biased design of the CPP testing was assessed as a possible explanation for the unexpected CPP results. Calculations for a CPP score were initially defined by the difference in time spent in the nonpreferred chamber, where animals were conditioned with cocaine, at the posttest compared to the pretest. Animals treated with saline only were expected to have CPP scores close to zero given that they were not conditioned to associate either chamber with the reinforcing properties of cocaine. After calculating average CPP scores that were equivalently high for all animals treated with cocaine and saline only, subsequent analysis of the raw data revealed that pregnant animals tended to form extreme preferences for one chamber or another in the pretest. This is problematic for the calculation of a CPP score because if these extreme preferences are inconsistent, then we might expect that animals conditioned with saline only

would either have a small negative or a large positive CPP score based on whether they are maintaining or switching their preference on the posttest. What was observed were inconsistent side preferences, creating artificially high, positive CPP scores even in the absence of cocaine. In an attempt to mitigate some of these issues, a secondary pretest was implemented in experiment 2b to account for our finding that pregnant animals' initial pretest chamber times may not accurately represent true side preferences. Preliminary data has shown that a secondary pretest may be more indicative of animal side preferences. When calculating CPP scores for saline only animals treated with vehicle using a secondary pretest, CPP scores were substantially closer to zero compared to calculating a CPP score using a primary pretest. A larger sample size is required to see if this methodological change better reflects motivation for cocaine using a secondary pretest for CPP score calculation.

Based on the described issues regarding CPP scores in saline only control animals, it is difficult to draw conclusions regarding the effect of ICI compared to vehicle on motivation for cocaine. In order to see any effects of estrogen receptor activation, animals that were not conditioned with cocaine must display an expected CPP score close to zero that differs from scores for animals conditioned with cocaine. This would allow for a comparison of CPP scores for animals treated with ICI or vehicle and conditioned with cocaine. Without considering these issues, the data suggest that estradiol acting within the brain does not impact motivation for cocaine. Previous findings do not support this conclusion because ICI has previously disrupted the formation of a CPP for cocaine in nonpregnant rats, suggesting that blocking estradiol may decrease motivation for cocaine (Segarra et al., 2014). Similarly, ovariectomized mice treated with estradiol showed an increased CPP for cocaine (Satta et al., 2018). Though these studies differ from the current study in that the hormonal manipulation occurred in nonpregnant animals,

together the findings do not agree on the impact of estrogen activation in the brain on motivation for cocaine. However, as previously mentioned, it is not fully justified to interpret the effects of estradiol given the issues with the CPP control data.

Furthermore, measuring the weight of both pregnant and nonpregnant animals was important in assessing the pregnancy status of the animals. The percent weight change was calculated between the pretest and posttest for each animal as an indicator of this measure. Pregnant animals in experiment 1 displayed an expected increase in mean body weight that was significantly higher than nonpregnant animals. This typical weight progression has also been observed in prior studies. For example, pregnant female rats had a significantly higher percentage of body mass compared to nonpregnant animals at days 4, 8, 10, and 18 of their pregnancy (Stramek et al., 2019). Pregnancy status was then confirmed through parturition for pregnant animals. In this same experiment, nonpregnant animals displayed a slight increase in percent body mass, similar to the nonpregnant animals in experiment 1. These data suggest there is confidence that percent weight gain was a reliable measure for monitoring pregnancy status.

Interestingly, animals treated with ICI had a higher change in weight from baseline at both the pretest and posttest compared to animals treated with vehicle. These findings highlight how estradiol may work in the brain to limit weight increase through mechanisms such as appetite suppression. Although studies on the effect of estradiol on food intake have not been investigated within pregnant animals, in nonpregnant female rats that were ovariectomized and treated with an ER-alpha agonist, there was a decrease in food intake compared to controls (Geary & Asarian, 2001). Looking further into this inhibitory mechanism, researchers found that estradiol may interact with glucagon to initiate satiation. Female rats treated with estradiol and glucagon displayed significantly less food intake than animals treated with oil and glucagon

(Geary & Asarian, 2001). In this regard, it would be expected that blocking estradiol in the brain through ICI would reduce this effect as measured by the higher change in weight compared to animals treated with vehicle. Given that the present study did not measure food intake nor energy expenditure directly, it is not possible to specifically attribute the observed weight change to either process. But it is interesting to also note that animals treated with ICI traveled less than animals treated with vehicle, implicating energy expenditure as a potential underlying mechanism (discussed in more detail below). It is also possible that the observed weight differences by hormone condition may be accounted for by estradiol's potential impact on pregnancy-related outcomes such as fetal size or body fat accumulation. Further studies incorporating additional physiological and metabolic measures are necessary to better understand the mechanism through which estradiol suppresses excessive weight gain in pregnancy.

Distance traveled was measured because CPP score is dependent on movement between the two chambers. Animals treated with saline only traveled less at the posttest compared to the pretest in experiment 1. Similarly, studies have used the number of ambulations to measure locomotive behavior during repeated exposure to an apparatus. In one study, researchers observed a significant decrease in ambulations between day 1 and day 7 of behavioral testing for rats treated with saline and placed in an initially novel environment (Martinez et al., 2019). Together, these results suggest that when an animal is not exposed to a drug of abuse and is repeatedly placed in the same apparatus, there is a decrease in exploratory behavior. On the other hand, cocaine treated animals did not show this decreased distance traveled at the posttest compared to the pretest. It is important to note that animals do not receive an injection of cocaine at the time of the posttest (last cocaine injection was received 48 hours prior); when considering this, one possible explanation is that animals with a prior history of cocaine treatment may

display high exploratory behavior at the posttest because they are motivated to seek cocaine within the chamber previously associated with this drug. The exploratory behavior explanation is further supported given that at the posttest, animals treated with saline only traveled less than animals treated with cocaine. Saline only animals would not be expected to be motivated to seek particular stimuli within the apparatus given that they receive no cocaine exposure. More detailed analyses of where the increased locomotor activity happens in cocaine treated animals, and how that compares to saline conditioned animals, would be useful to better understand if the sustained, high locomotion in cocaine treated animals at the posttest is due to exploratory behavior in the cocaine-paired chamber.

As discussed earlier, saline only animals in experiment 2 treated with vehicle traveled more than saline only animals treated with ICI. These findings suggest that estradiol may act in the brain to increase the distance traveled by pregnant animals. The existing literature on estradiol's impact on locomotion supports this conclusion. Estradiol has previously been found to increase locomotor activity in ovariectomized, female rats who displayed increased distance run on a running wheel when treated with estradiol compared to oil (Espinosa & Curtis, 2018). Interestingly, these animals treated with estradiol also displayed higher levels of dopamine in the nucleus accumbens which introduces a potential mechanistic explanation for estradiol's impact on locomotion. Estradiol has been shown to increase dopamine levels by potentially reducing GABA's inhibition on dopamine release (Schultz et al., 2009). The literature has previously identified dopamine's role in locomotion using rodent models with motor deficits. In one study, increased locomotion was observed by optogenetically activating medium spiny neurons containing dopamine receptors in the nucleus accumbens as part of the basal ganglia circuitry (Kravitz et al., 2010). Together, these data suggest that estradiol may increase locomotion

through its impact on dopamine release in the brain. However, data for cocaine treated animals do not fit with this explanation given that ICI animals showed enhanced locomotion. Additional studies will be required to investigate the effect of estradiol on locomotion in animals with a history of cocaine exposure.

In conclusion, while pregnant animals did form a comparable CPP for cocaine to nonpregnant animals, the follow-up hypothesis regarding estradiol's impact on motivation for cocaine in pregnant rats could not be definitively tested due to issues with CPP scores in control animals. Future directions include expanding the sample size of animals that are pair-housed and scored using a secondary pretest as described in the methodology for experiment 2b. There is also room to expand upon the secondary results to look at areas such as the mechanism behind estradiol's impact on weight gain during pregnancy and analysis of where high locomotion of cocaine treated animals occurs within the apparatus.

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