GESTATIONAL COCAINE: EFFECTS ON POSTPARTUM BEHAVIORS AND ENDOCRINE SIGNALING IN A RODENT MODEL

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ABSTRACT

SARAH K. WILLIAMS: GESTATIONAL COCAINE: EFFECTS ON POSTPARTUM BEHAVIORS AND ENDOCRINE SIGNALING IN A RODENT MODEL
(Under the direction of Josephine M. Johns)

Cocaine use by human mothers during pregnancy is highly correlated with child neglect, maternal anxiety and depression. Human and rodent infants exposed to cocaine *in utero* exhibit altered behavioral and neurological phenotypes, and appear to be less able to elicit normal care from biological and foster mothers. In rodents, specific pup-produced stimuli, including vocalizations, olfactory cues, and body temperature, have been associated with the initiation and maintenance of maternal response. Animal models indicate that chronic cocaine (CC) treatment throughout gestation disrupts postpartum maternal behavior (MB), including retrieving pups to the nest, nursing and licking behaviors, while simultaneously altering oxytocinergic signaling (receptors, levels, synthesis). It has not yet been determined if plasma levels of OT in rodents are also altered by CC treatment or if they are associated with brain region-specific changes.

The current studies found that gestational cocaine exposure alters the retrieval behavior of dams in an interactive manner depending on dam treatment, pup treatment and postpartum day tested. On PPD5, CC-treated dams were found to have lower plasma OT, without differing in brain OT following a retrieval test. Group pup ultrasonic vocalizations did not differ significantly between prenatal exposure conditions, indicating these may not be the most relevant cues by which dams differentiate between litters. In addition to changes in response to pups, CC treatment resulted in disrupted stress-coping that was associated with increased hormonal stress responsiveness and changes in brain OT levels, both of which could be a mechanism of altered maternal response. Olfactory preference for pup urine declined across the postpartum period and

only CC-treated dams specifically avoided CC-exposed pup urine olfactory cues. The complex nature of maternal-infant interactions during the early postpartum period was clear in this study, highlighting the need for continued studies to pinpoint the underlying mechanisms of cocaine-induced changes in dam maternal response and how early differential patterns of biological and behavioral measures in pups might further exacerbate disruptions in maternal care by any dam. Furthermore, these studies suggest that stress-responsiveness may be an important contributing factor to disruptions in cocaine-induced deficits in MB.

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LIST OF ABBREVIATIONS

AHN	anterior hypothalamic nuclei
AMY	amygdala
ANOVA	analysis of variance
AOB	accessory olfactory bulb
BNST	bed nucleus of the stria terminalis
CC	chronic cocaine
CoA	cortical amygdala
CORT	cortisol/corticosterone
CPP	conditioned place preference
CS	chronic saline
DA	dopamine
FST	forced swim test(ing)
GD	gestation days
HIPP	hippocampus
HPA	hypothalamic-pitutary-adrenal
MB	maternal behavior
MeA	medial amygdala
MOB	main olfactory bulb
mPFC	medial prefrontal cortex
MPOA	medial preoptic area

NAc	nucleus accumbens
NE	norepinephrine
OD	optical density
OFT	open field test(ing)
OPT	olfactory preference testing
PAG	periaquaductal gray
PBS	phosphate sodium buffer
PC	prenatal cocaine
PFA	paraformaldehyde
PND	postnatal day
PPD	postpartum day
PVN	paraventricular nucleus
SON	supraoptic nucleus
UN	untreated
USV	ultrasonic vocalization
UTI	urinary tract infections
VMH	ventromedial hypothalamus
VTA	ventral tegmental area
5-HT	serotonin

CHAPTER 1. INTRODUCTION

1.1. COCAINE USE DURING PREGNANCY: A PUBLIC HEALTH CONCERN

Illicit drug use during pregnancy has been historically related to a number of anomalous effects on behavioral and neurobiological measures in offspring exposed during fetal development, but even so, approximately 5% of pregnant women still reported illicit substance use as late as 2006 (Office of Applied Studies 2008). It was estimated that prenatal cocaine (PC) exposure increased the number of children who needed special educational services by as many as 80,550 per year during the 1990's, costing billions in educational and medical services (Lester et al. 1998, Behnke et al. 1998, Behnke et al. 1997, Frank et al. 2001). There is an abundance of literature documenting both physiological and behavioral changes following PC exposure, which has been the subject of several earlier reviews (Glatt et al. 2000, McMurray et al. 2008, Stanwood et al. 2001, Bakshi et al. 2009, Chae & Covington 2009, Delaney-Black et al. 2000, Spear et al. 2002, Meyer & Zhang 2009, Ho et al. 1994, Chiriboga et al. 1999, Olsen & Murphey 1995). These behavioral problems can be exacerbated by stressful or neglectful home environments, more typically found in homes where parents abuse drugs (Murphy et al. 1991, Eiden et al. 2007).

1.2. COCAINE USE DISRUPTS PARENTING IN CLINICAL POPULATIONS

Relatively less research has focused on the impact of drug exposure during pregnancy on maternal mental health and behavior, although a recent review highlights the literature and the areas that need future exploration (Strathearn & Mayes 2010). Maternal cocaine use during pregnancy has been correlated with a greater incidence of maternal neglect (Kelley 1992),

problems with mother-infant bonding (Burns et al. 1991), and child abuse (Murphy et al. 1991). Cocaine-using mothers exhibit lower attentiveness and responsiveness toward their infants (Mayes et al. 1997, Eiden et al. 2011), and are more likely to express hostility during feeding and play interactions (Goldman-Fraser 1997, Light et al. 2000), with deficits for many women lasting at least through the toddlerhood of their children (Molitor & Mayes 2010). These behaviors result in a 20-fold increased likelihood that the child will be removed from the mother (Eiden et al. 2007). Clinical studies have begun to examine neurobiological factors thought to be important for maternal response, demonstrating that compared to non-using mothers, drug-abusing women have lower plasma levels of oxytocin (OT) and cortisol (CORT), hormones known to be involved in maternal response and stress (Light et al. 2000, Light et al. 2004, Schuetze et al. 2003, Slattery & Neumann 2008). Cocaine use, depression and anxiety are highly correlated in clinical populations (Rounsaville 2004, Hans 1999), specifically in postpartum women (Singer et al. 1995, Hans 1999). Mood disorders can negatively impact maternal-infant interactions independently of drug use (Smith et al. 2004, Braw et al. 2008, Leahy-Warren & McCarthy 2007) and may contribute to disrupted maternal-infant interactions in drug-abusing women. Additionally, most human studies use patient populations who are polydrug users (LaGasse et al. 2003, Singer et al. 1995), making mechanistic studies of the specific effects of cocaine use difficult in human populations. Additionally, understanding deficits in maternal behavior (MB) are complicated by the variety of factors influencing MB in postpartum women (see Figure 1).

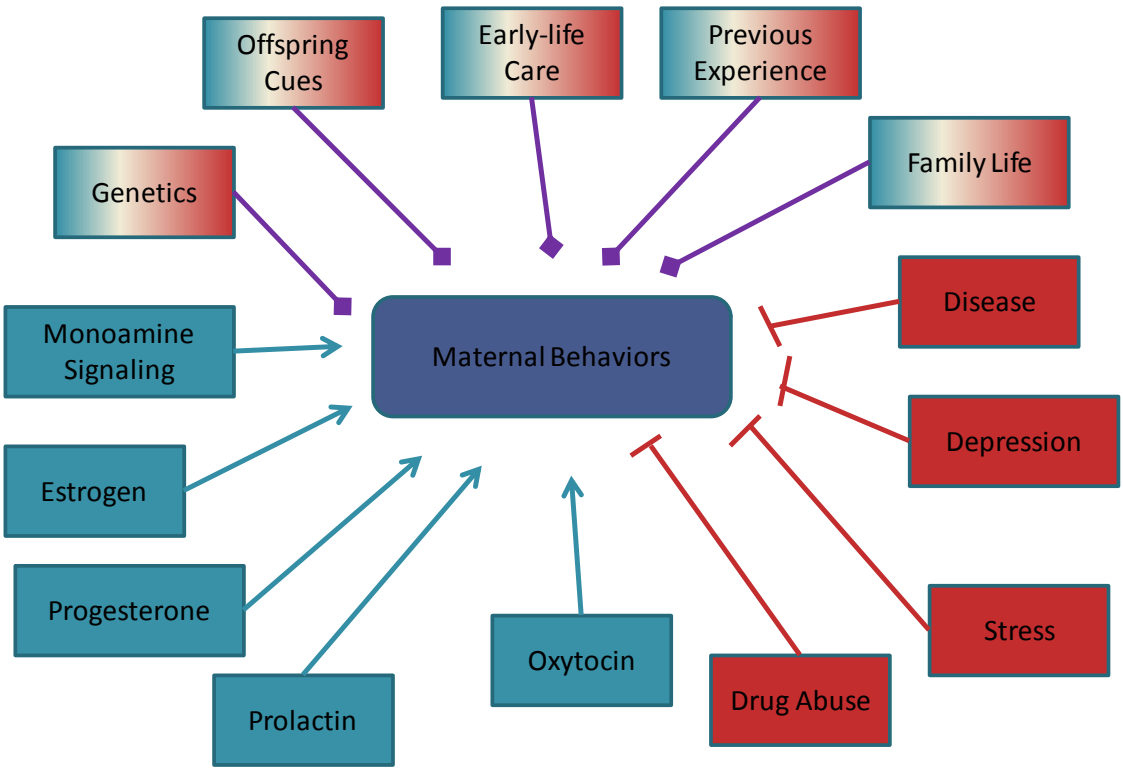

Figure 1. Maternal Behavior Influences.

The initiation and maintenance of maternal behavior is promoted by increases in a number of neurobiological and endocrine factors (teal boxes). MB can be disrupted by many variables (red boxes). Additionally, several factors can either promote or disrupt optimal MB in a context-specific fashion (multi-colored boxes). The combination of these factors contributes to the behaviors exhibited by the mother as she continues through the postpartum period. MB: maternal behavior

1.3. PRENATAL COCAINE EXPOSURE ALTERS HUMAN INFANT BEHAVIOR RELEVANT TO CARE

Maternal-infant interactions require the participation of both parties, including infant behavior. Therefore, PC exposure-induced changes in behavior may elicit different reactions from mothers. PC-exposed human infants show quicker and greater frustration with tasks compared to control infants (Eiden et al. 2009, Chaplin et al. 2009). This is especially interesting given that another study found PC-exposed toddlers showed less negative reactivity to separation from their mothers, typically considered a stressful event (Molitor et al. 2003). PC-exposed infants differ in their response to the Still-Face task, a measure of interpreting social cues (Lewis et al. 2009). PC-

exposed toddlers consistently show less reactivity to separation from their mothers (Molitor et al. 2003) and as children, are less empathic. Additionally, they exhibit greater frontal cortical asymmetric activity compared to non-exposed children when shown a crying infant or their own mother (Jones et al. 2004), supporting the hypothesis that alterations in frontal cortex function plays a role in behavioral disruptions.

Although PC exposure is associated with adverse effects on the infant, the role of those effects as they relate to early mother-infant interaction and a child's ability to elicit care normally from a mother is largely unknown. The highly interactive nature of mother and infant, during the postpartum period when physiological changes associated with parturition and early attachment behaviors develop, makes it extremely important to gain an understanding of drug effects on both mother and infant during this period (Dow-Edwards 2011).

Rodents serve as excellent preclinical models for the study of onset and maintenance of MB, because like humans, they produce altricial infants requiring an immense commitment in order to ensure offspring survival. The typical rat mother (or dam) will spend the majority of her time in contact with pups for two weeks postpartum, only leaving to forage for food. Rodents exhibit stereotyped behaviors toward pups that can be easily quantified and have operationally defined behavioral correlates to humans, including nursing and grooming, as well as preparing a safe environment for the infant (nest-building).

1.4. ANIMAL MODELS DEMONSTRATE COCAINE-INDUCED DEFICITS IN MATERNAL-INFANT INTERACTIONS

Studies on mother-infant dynamics in the rodent have shown that treatment with cocaine, through acute, intermittent, or chronic treatment regimens, disrupts aspects of MB, with the extent of disruption dependent on dose, duration and time of testing, and treatment regimen used (Johns et al. 1994, Nelson et al. 1998). In such studies, dams received cocaine either chronically on gestational days (GD) 1-20 (30 mg/kg) or acutely (30 mg/kg) via a single subcutaneous injection immediately following parturition and were then tested for pup-directed MB following a

brief separation and reunion with pups (Johns et al. 1994). Both acute and chronic treatment led to increased latency and decreased duration of nursing, along with disruptions in licking and nest-building. Later studies found these disruptions following chronic gestational treatment did not appear to result from hyperactivity or cocaine withdrawal (Johns et al. 1997). In agreement with clinical studies that found lower plasma OT levels associated with cocaine use and maternal response differences (Light et al. 2004), cocaine treatment in rat dams was correlated with lower OT levels in the early postpartum period (Johns et al. 1997), specific to brain regions associated with MB onset. Biologically relevant levels of OT are not thought to cross the blood-brain barrier easily (Kang & Park 2000), although discussion on this point is continuing (McEwen 2004); presently it is unknown if plasma levels are similarly altered in cocaine-treated animals as in humans. If so, it would highlight additional peripheral effects of cocaine-induced changes in OT as possible mechanisms of interest.

1.5. MATERNAL NEUROCIRCUITRY RELATIVE TO MATERNAL RESPONSE TO INFANT CUES

In order to initiate and maintain the complex behaviors involved in maternal care, dams must sense, perceive, integrate, assign value and initiate responses to an infant, tasks that involve a variety of neurological systems. The neural circuitry involved in maternal response and behavior has recently been reviewed, and includes and overlaps significantly with those circuits involved with reward, stress, executive function and somatosensation (see Appendix A; Figure 27 and (Numan 2007)). Multiple reviews have proposed that dopamine (DA), thought to have a prominent role in drug reward, acts as a critical signal in the "reward circuit", consisting of the midbrain-forebrain pathway that connects the ventral tegmental area (VTA) with the nucleus accumbens (NAc) and medial prefrontal cortex (mPFC). This pathway allows information converging in the NAc to drive locomotor responses to seek rewards (Koob & Volkow 2010, Sesack & Grace 2010), and is believed to be responsible for motivating action toward more natural rewards such as food, sex, and importantly to our discussion, maternal care (Ikemoto

2007).

1.6. REWARDING VALUE OF PUPS AND PUP CUES: BEHAVIORAL DATA

Pups and pup-produced cues have been shown to have rewarding (motivational) value to rat dams, measured with both conditioned place preference (CPP) and operant responding models. The reinforcing value of pups to dams depends on an interaction between the postpartum age of the dam and the postnatal age of the pups (Wansaw et al. 2008). Although pups of many ages will induce CPP, younger pups induce greater conditioning than older pups, although CPP decreases across the postpartum period regardless of pup age (Wansaw et al. 2008). Additionally, like conditioning of drug-related cues, pup-associated cue conditioning is dependent on the amount of separation time from the cue and length of testing, indicating that learning about reinforcing cues is not altered in the postpartum period (Wansaw et al. 2007, Pereira & Morrell 2009). Of particular note, pups often induce greater CPP than cocaine, indicating the strength of motivational salience pups possess (Mattson & Morrell 2005, Mattson et al. 2003, Seip & Morrell 2007). However, approximately 30% of rat dams do not show a preference for pups over cocaine, indicating inherent variability to sensitivity to pup cues' rewarding value. The underlying neurobiology of variability in this behavior is not completely understood.

1.7. HORMONAL REGULATION OF MATERNAL BEHAVIOR

A variety of endocrine signaling cascades maintain and signal the end of pregnancy, and initiate lactation, producing changes in both physiology and neurobiology (Weiss 2000, Brunton & Russell 2008). These endocrine systems include estrogen, progesterone, prolactin, CORT and OT (see Figure 2). These systems interact with each other to allow for a smooth transition between pregnancy, parturition, and the postpartum period. Plasma OT is essential for milk ejection, uterine contractions and is associated with stress (Hashimoto et al. 1989, Kalin et al. 1985, Light et al. 2004).

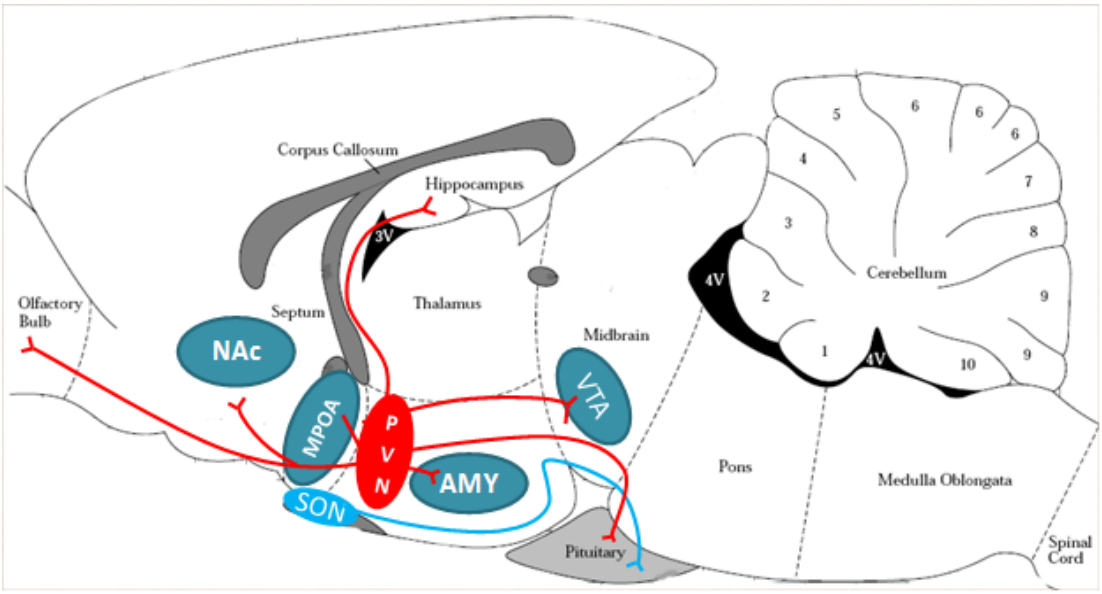

Figure 2. Oxytocinergic Projections.

Magnocellular OT cells in the PVN and SON project to the posterior pituitary for peripheral release for lactation and stress response. Parvocellular cells in the PVN project to areas of the 'reward' circuit (VTA, NAc), the stress circuit (PVN, AMY, hippocampus), as well as to somatosensory cortices (olfactory bulbs, auditory cortex). Projections also reach the MPOA which is a major organization center for MB. These projections allow OT to influence behavior in a variety of ways during MBs. PVN (paraventricular nucleus), SON (supraoptic nucleus), VTA (ventral tegmental area), NAc (nucleus accumbens), AMY (amygdala), MPOA (medial preoptic area).

Both plasma and brain OT can interact with hypothalamic-pituitary-adrenal (HPA) axis activity (Windle et al. 1997, Gibbs 1986); This may have implications for human clinical research, as one human study recently reported that mothers who used cocaine during pregnancy had reduced plasma OT levels and correlated dysregulation of the stress response associated with altered maternal response to infants (Light et al. 2004).

Central levels of OT play a critical role in the onset of rat MB and are likely involved in the maintenance of MB, the extent and direction of which is probably dependent on day of testing. The onset (postpartum day (PPD) 1-4) and maintenance periods (PPD 5-21) of MB are differentiated by their underlying hormonal state (Insel et al. 2001). The presence of OT is critical to initiating the onset of MB in several mammalian species including the rat (Pedersen & Prange 1987, Kendrick et al. 1987, Van Leengoed et al. 1987, Lee et al. 2009, Nemsadze & Silagava

2010), but its role in maintenance of MB is less clear. In the rodent, OT neurons from the paraventricular nucleus (PVN) of the hypothalamus project to the posterior pituitary for release into the peripheral circulation, and centrally to the medial preoptic area (MPOA), main olfactory bulb (MOB), NAc, amygdala (AMY), hippocampus (HIPP), and VTA (see Figure 2). Many of these regions mediate behavioral responses relevant to maternal interactions. OT levels in the supraoptic nucleus (SON), PVN and plasma are highly correlated; however, the relationship between plasma levels of OT and other brain region-specific levels is still unknown (Wotjak et al. 1998). OT processes from the SON project to the pituitary for peripheral release into the bloodstream in response to infant-produced or stressful stimuli (Wotjak et al. 1998). OT is known to bi-directionally interact with HPA activity, with chronic OT treatment leading to reduced acute stress responses (Uvnas-Moberg et al. 2005), suggesting that OT may mediate hypoactive stress response in the postpartum period (Slattery & Neumann 2008).

In addition to the OT stress response in mothers, the gestational and postpartum periods are characterized by high basal CORT levels, a hypo-responsive hormonal reaction to stress and low anxiety levels (Slattery & Neumann 2008). Changes in maternal stress responses have been correlated with deficits in maternal care (Bosch et al. 2007, Chen et al. 2010, Smith et al. 2004). Stress during pregnancy can reduce MB in rodents, however, stress did not affect the rats that had low baseline MB, suggesting that optimal care can be reduced only to a certain extent (Champagne & Meaney 2006). Administering CORT to pregnant or lactating rats decreases nursing and increases neglectful behaviors (Bosch et al. 2007, Brummelte & Galea 2010). Repeated stressors during the postpartum period can also inhibit lactation in rodents, suggesting direct hormonal effects (Lau & Simpson 2004). Conversely, removing circulating stress hormones reduces, but does not abolish, MB (Rees et al. 2004). Although it is clear that cocaine can disrupt stress response (Goeders 2002) few studies have tied differences in stress response (CORT) to cocaine-induced deficits in rodent MB or maternal-infant interaction.

1.8. GESTATIONAL COCAINE DISRUPTS OXYTOCIN IN THE POSTPARTUM

Early studies proposed that cocaine may modulate MB in the rat in the early postpartum period through its effects on the OT system (Johns et al. 1994). OT is disrupted by cocaine in several regions, in parallel with behavioral disruptions of MB onset or aggression (Johns et al. 1994, Johns et al. 1998, Johns et al. 1997, Johns et al. 2004, Johns et al. 2005). Gestational chronic cocaine (CC) treated rat dams had significantly lower OT levels in the MPOA, HIPP and VTA within 24 hours of delivery (Johns et al. 1997) (see Figure 3). In rats, these brain regions normally require functional OT systems for initiation of MB to occur (Pedersen et al. 1994, Numan & Stolzenberg 2009). Furthermore, CC treatment results in decreased OT levels during mid-lactation (PPD 5-10) in the amygdala, which has been tied to changes in maternal aggression (Johns et al. 1995, Johns et al. 1998, Lubin et al. 2003).

Although the onset of MB is largely dependent on changes in endocrine system function (Numan & Insel 2003, Keverne 1988, Nemsadze & Silagava 2010, Brunton & Russell 2008); somatosensation, olfaction, and infant-produced auditory stimuli are clearly important for maternal response across the postpartum period in rats as reviewed below. In a series of intergenerational studies of MB following cross-fostering of pups, it was shown that CC-exposed infant rats were less capable of eliciting maternal care from control as well as drug-treated dams (Johns et al. 2005).

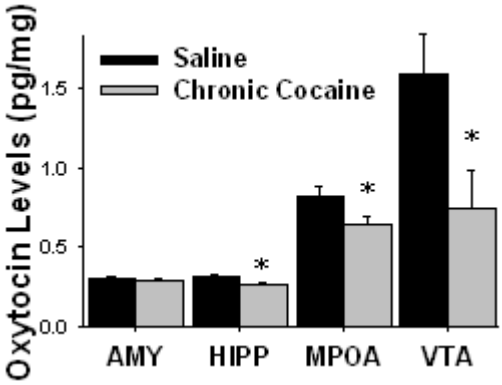

Figure 3. Brain Oxytocin Levels Affected by CC treatment.

Data presented as means ± SEM. CC dams have lower OT levels at MB onset in MPOA, VTA, and HIPP compared to CS dams. $p \leq 0.05$ (Student's t test). CC: chronic cocaine; OT:oxytocin; MB:maternal behavior; AMY: amygdala; HIPP: hippocampus; MPOA: medial preoptic area; VTA:ventral regmental area.

Although, it is clear that pups can elicit maternal responses, it is still unclear which pup-produced cues are the most effective at eliciting care (Farrell & Alberts 2002, Smotherman et al. 1978), and if these cues differ in CC-exposed rat pups. In addition, gestational CC treatment may disrupt the ability of dams to distinguish between different aspects of pup-produced cues.

1.9. PUP-PRODUCED CUES RELEVANT FOR MATERNAL CARE

MB has long been associated with control through multisensory systems and this remains the dominant theory in the field (Numan & Insel 2003, Stern 1990, Stern 1997, Smotherman et al. 1978, Farrell & Alberts 2002). Pups provide thermal, sensory and suckling cues to the dams, suggesting that no single sensory system may be imperative for the initiation or maintenance of MB in rats, but rather a combined set of stimuli function in this capacity. Somatosensory cues (touching of dam by pups) clearly play a strong role in nursing (or crouching) behavior, as dams will retrieve, lick and mouth dead or chilled pups, but not crouch over them (Stern & Lonstein 1996).

1.9.1 Auditory Cues

Infant rat pups emit ultrasonic vocalization (USVs), believed to be similar in attention-eliciting function to cries emitted by human infants. The temporal threshold for rodents to distinguish and behaviorally respond to complex socially-relevant sounds from background noise overlaps exactly with the normal temporal spacing of infant USVs (Ehret 2005). Specifically, dams have increased electrophysiological activity in the left dorsoposterior field of the auditory cortex compared to virgin mice, which seemed to reflect the increased integration of call recognition (Fichtel & Ehret 1999, Geissler & Ehret 2004).

The effect of developmental exposure to drugs of abuse on USVs varies by drug, timing and dose of exposure, with mixed results for early cocaine exposure being reported. Neonatal exposure to cocaine (postnatal days (PND) 4-11) has been reported to cause both increases and decreases in isolation-induced USVs on PND 14 (Barron et al. 2000, Barron & Gilbertson 2005), while PC exposure decreased USVs in mice (Hahn et al. 2000). The effects of PC exposure on USVs produced by a litter of pups, which is the most ethologically common stimulus situation for maternal-infant interaction in rodents, has never been investigated, nor has the impact of litter differences in USV production on MB been reported.

1.9.2 Olfactory Cues

Olfactory cues influence MB in many mammalian species (Levy et al. 2004), including human mothers who can detect subtle olfactory cues from babies (Bonnin et al. 1990). Pup-produced olfactory cues are primarily thought to be from urine, feces or the preputial gland excretions. These excretions are released by maternal licking in the early postnatal period and rat dams consume pup urine for their own water and electrolyte balance (CAPEK & Jelinik 1956, Friedman et al. 1981, Gubernick & Alberts 1983). Although olfactory cues are important to dams in the postpartum period to identity and confirm the health of a pup, information about how urine may serve as a stimulus cue during the postnatal period remains extremely sparse. PC exposure

may alter urine composition and odor and thus impact a potential signaling system to the mother.

1.10. SUMMARY

Cocaine treatment or use results in abnormal maternal-infant interactions during the postpartum period in humans and rodents. The mechanisms underlying these behavioral effects are unknown, but evidence from the preclinical literature and preliminary evidence from human studies suggest that drug effects on both the mother and infant contribute to the overall deficits. This body of evidence led to this investigation of the effects of gestational cocaine exposure on specific aspects of maternal response and infant cues as well as basic neurobiological measures as possible mechanisms in a rodent model. Predictions of outcomes, which were based on previous research findings, were that CC-treated dams would exhibit reduced MB, operationally defined as slower or less pup retrieval, and little or no preference for spending time in a chamber containing pup olfactory cues. As measures of stress and distress, also thought to affect MB, disruptions in endocrine levels and signaling in CC-treated dams were expected. Finally as part of the interaction dyad, it was hypothesized that CC-exposed pups would contribute to altered maternal response through altered USV and olfactory cue production.

CHAPTER 2. AIMS, GENERAL METHODS AND PROCEDURES

2.1. SPECIFIC AIMS

Specific Aim I: a. To determine if CC treatment affects maternal retrieval response and pup choice, independent of the effects of pup CC exposure; b. To determine if CC-exposed pups elicit less MB from all dams compared to non-exposed pups; and c. To determine if pup litter USVs correlate with maternal retrieval.

Hypotheses: CC-treated dams will exhibit slower retrieval compared to CS and UN dams, CC-exposed pups will be retrieved later and elicit poor MB from all dams, and this will be related to the smaller number and shorter duration of pup litter USVs.

Specific Aim II: a. To determine if maternal response to pups is correlated with peripheral and central OT level differences in CC-treated versus control (CS, UN) dams; b. To determine whether plasma OT and specific brain region OT levels correlate in postpartum dams and if this is different in CC dams.

Hypotheses: CC-treated dams will have lower plasma and brain region OT levels than control dams. OT levels in the hypothalamus, VTA, and AMY will correlate with levels in the plasma (i.e. high plasma level, high brain region level).

Specific Aim III: a. To determine if CC-treated dams compared to CS and UN dams differentially prefer pup-produced olfactory stimuli (urine) from CC versus UN and CS prenatal exposure conditions. Additionally, to determine if there are differences in urine constituents of CC pups compared to control pups as a possible mechanism of preference differences b. To determine if CC-treated dams exhibit differential neuronal activation (c-Fos) patterns in response

to pup olfactory stimuli from CC-exposed and UN pups.

Hypotheses: All dams will show preference for UN pup urine on PPDs 1 and 3. On PPD5, UN and CS dams will prefer their own pups' urine, but CC dams will not. CC-exposed pup urine constituents that can affect urine odor (ketones, glucose, bilirubin, proteins) will differ from control pup urine. CC-treated dams will show lower c-Fos expression in response to pup urine in the NAc compared to UN dams.

Specific Aim IV a. To determine if CC-treated dams differ in measures of anxiety or stress in the postpartum period; b. To determine if HPA axis function (through OT and CORT measures) differ in CC-treated dams compared to UN dams following behavioral testing; and c. To determine if OT in specific brain regions is correlated with stress-responsive behaviors of CC and UN dams.

Hypotheses: CC-treated rat dams will exhibit higher anxiety and depressive-like or stress-related behavior on PPD5 compared to UN dams. CC dams will have higher baseline and post-behavior CORT blood levels. OT levels will be higher in the hypothalamus and AMY of CC-treated dams compared to UN dams and OT levels in the hypothalamus will be correlated with CORT levels in all dams.

2.2. GENERAL METHODS FOR ALL AIMS

2.2.1 Subjects

Sprague-Dawley nulliparous female rats (~200 grams, Charles River, Raleigh, NC) were kept on a 12:12 reverse light cycle (8:00 AM dark) for at least one week and then mated with a single male until conception was noted by the presence of a vaginal plug or sperm in a vaginal smear and defined as GD 0. Seven days following conception (GD7) females were moved to a colony room and individually housed on a regular 12:12 light cycle (7:00 PM dark). This procedure typically results in 95% of the females delivering during the afternoon hours (Mayer & Rosenblatt 1998). Females were randomly assigned to treatment or control groups of 9-15 each,

as they become pregnant. Gestational weight gain was measured daily for all groups. Water and chow was available ad libitum for all but CS-treated rat dams who were matched with CC-treated dams on a pair-feeding schedule (see Methods, Chronic Saline). Breeding was staggered to allow approximately one dam from each treatment to be tested on the same day. Postpartum day (PPD) 1 was defined as within 12-18 hours following completed delivery. Immediately following parturition, pups were removed from the dams, and gestational length, litter weight, number of pups per litter, and sex ratio were recorded. Dams reared a culled litter of ten of their own biological pups (as close to 5 male/5 female as possible).

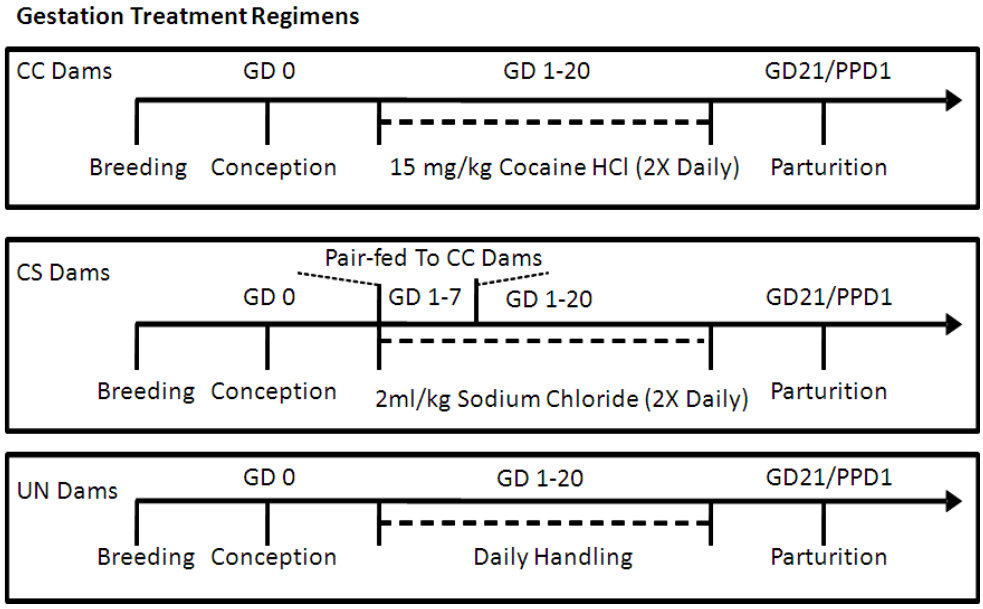

Figure 4. Gestation Treatment Regimens.

Dams were divided into 3 possible treatment conditions that lasted throughout gestation and ended the day before parturition. CC: chronic cocaine. CS: chronic saline. UN: untreated. GD: gestational day. PPD: postpartum day.

2.3. TREATMENT REGIMEN

2.3.1 Chronic Cocaine

Dams received 30 mg/kg/day of cocaine HCl (dose calculated as free base, 2 ml total volume, Sigma, St. Louis, MO) in a saline solution. This is the lowest dose at which we consistently find significant effects on MB (Johns et al. 1994, Johns et al. 1998). Half of the total cocaine dose (15 mg/kg) was injected twice daily, subcutaneously, at approximately 9:00 AM and 4:00 PM throughout gestation (GD 1-20) and not thereafter. We have used acute, intermittent and chronic dosing schedules either ending on GD 20 or extending into the postpartum period and determined that this schedule was the best for this project (Johns et al. 1997). The absorption rate of the subcutaneous injection route is relatively analogous to "snorting" cocaine by humans (Spear et al. 1989) and our dose (15mg/kg in rat) is equivalent to 1 gram of cocaine in a 156 pound woman. The use of a 27.5 gauge needle and rotating injection sites significantly reduces the number and severity of cutaneous lesions frequently reported with CC injections in rat models. A topical antibacterial ointment (Polymycin-Bacitracin-Neomycin, Glaxo-Wellcome, Raleigh, NC) was applied on any skin lesions as they were discovered. Subcutaneous administration of cocaine should not cause significant behavioral or biochemical stress when carefully monitored (National Institute on Drug Abuse 1993).

2.3.2 Chronic Saline (CS)

Dams received an injection of normal saline (0.9%; 1 ml/kg) subcutaneously twice daily throughout gestation (GD 1-20) at approximately 9:00 AM and 4:00 PM based on daily weights. Dams were pair-fed to CC-treated dams during early gestation (GD 1-7) such that the amount of food CC-treated dams ate on average on a specific GD was the amount provided to CS-treated dams on the corresponding gestational day. Beginning on GD 8, CS-treated dams had free access to rat chow. This procedure has been found to account for the time when CC-treated dams are

most affected by the anorectic effects of CC (Delaney-Black et al. 1996), while not inducing the serious confound of food deprivation in CS-treated dams for the rest of gestation. Rapid weight gain does not occur for typical rat dams in the first week of pregnancy and CS-treated dams do not differ from UN controls or CC-treated dams (see Appendix F; Table 10), thus the food-yoking should not cause major malnutrition. However, it is necessary to include a CS treatment group for most behavioral measures, as there could be subtle differences in behavior resulting from injections or nutritional stress (Lubin et al. 2001, Tonkiss et al. 1995, Spear et al. 2002).

2.3.3 Untreated Controls

Dams received no drug treatment or food restriction during gestation or during the postpartum period, but were weighed daily to control for effects of handling.

2.3.4 Pup-Providers

Identical to dams in their same treatment group, UN pup providers received no treatment other than weighing and handling, and CC-treated pup providers were treated with cocaine as described above for CC-treated dams. The pup providers produced and reared culled litters of ten pups (5 male/5 female) for behavioral tests or urine provision (as indicated in relevant Specific Aims). CS pups were used on PPD 5 only (see Specific Aim I a, II b) as they were not expected to differ significantly from UN pups based on previous studies. Each test dam had two pup providers who gave birth on the same day assigned to her for the different tests requiring use of unfamiliar pups for behavioral testing. No dam was tested twice with the same pups.

2.4. GENERAL TEST PROCEDURES

Nine to fifteen dams were bred for each group of test dams needed for the experiments and pup providers were assigned to them (see Pup Providers). The same experimental dams were used in Specific Aims I, IIa & III. Groups of pup-provider dams were used for Specific Aim II b. A second group of pup provider dams was used for Specific Aim IV. Dams and their culled natural

litters were brought to the test room (28 °C, 70% humidity) on PPDs 1, 3, and 5 at approximately 09:00. For each test, side position and order of stimuli presentation were counterbalanced across groups and between sessions. After testing, dams and their litters were returned to the colony room until the next test session or immediately sacrificed.

2.4.1 Videotape Capture and Computer Analysis Method

A video recorder (using a Panasonic VHS (AG188U) or JVC recorder with low-light sensitivity) placed directly in front of the test chamber was started prior to testing and continued until the session ended. Every session began with a card to identify the animal PPD, and pups used. Cards were coded to hide the identity from scorers. Two independent scorers blind to treatment scored videotapes and their observations were assessed for reliability within 10% for frequency and latency and within 20% for duration of behaviors of interest. If the scores did not match initially, the sessions were rescored until reliability criteria were met. A computer program previously described (Johns et al. 1998, Nelson et al. 1998) recorded and calculated the frequency, duration, latency and sequence of behaviors displayed by the rat dams as the viewer scored the session.

2.4.2 Decaptitation

Rats for the endocrine experiments were euthanized by rapid decapitation. This method was employed such that the neuropeptide levels could be captured with dams experiencing as little stress as possible and to avoid alteration of neuropeptide levels by various anesthetics. Neuropeptide levels change rapidly in rodents and any behavioral stress can cause rapid release and lead to false reading of OT levels. All methods used standard procedures advocated by the UNC Division of Laboratory Animal Medicine and approved by UNC-Chapel Hill Institutional Animal Care and Use Committee.

2.5. IMMUNOHISTOCHEMISTRY

2.5.1 Transcardial Perfusion and Tissue Sectioning

Rats were brought to the procedure room and given a lethal dose of sodium pentobarbital. After pain assessment (toe-pinch), non-survival transcardial perfusion with 0.1 M phosphate buffer saline (PBS) solution was begun until blood was cleared. This was followed by 5 minutes of flushing with 4% paraformaldehyde (PFA) in 0.1 M PBS. Brains were removed, post-fixed in 4% PFA overnight and switched to PBS at 4º C. Following several days of 30% sucrose solution incubation, brains were frozen to -20 °C. 50 µm sections were cut using a cryostat (Leica 3500, Germany) and stored at -20ºC in ethylene-glycol-based cryoprotectant. Tissue was thawed once to select sections from brain regions of interest and returned to freezer until time of staining protocol.

2.5.2 Tissue Treatment

Tissue was removed from the freezer and thawed to room temperature. Sections were rinsed with PBS (x3). The inhibition of exogenous peroxidases was accomplished with 1% hydrogen peroxide. Brain sections were rinsed in PBS (x3). Sections were blocked with serum (10% normal goat serum plus Avidin-D (VectorLabs, Burlingame,CA)) overnight at room temperature. Sections were incubated with the primary antibody solution (Rabbit anti-c-Fos, SantaCruz Biotechnology + Biotin (VectorLabs Burlingame, CA)) for 48 hours at 4° C. Sections were rinsed with PBS (x3) and then exposed to the biotinylated secondary antibody (goat anti-rabbit (VectorLabs Burlingame, CA)) for 2 hours at 4° C. Sections were rinsed with PBS (x3). Avidin-Biotin Peroxidase Complex (Vectastain ABC kit, Vector Labs Burlingame, CA) was prepared 30 minutes before use, applied to sections, and allowed to incubate for 1 hour at room temperature. Sections were rinsed PBS (x3). The Diaminobenzidine (DAB: Polysciences, Warrington PA) reaction was performed. Sections were rinsed with PBS (x3), mounted and allowed to dry at room temperature overnight. Sections were then dehydrated with a series of alcohols and coverslipped.

2.5.3 Unbiased Stereology

Design-based stereology is a method of unbiased quantification of regional volume and/or total object-of-interest estimates that has recently seen increased use in the field of neuroscience (Schmitz & Hof 2005). When stereology is performed, an initial variance estimate is performed in which the goal is to count an average of 150-200 cells over approximately 100 counting frames for each structure (Mouton 2002). Following a pilot study to optimize counting parameters (50 x 50 um counting frames, 300 x 300 um grid size), it was determined from variance estimates that every seventh section would be measured (see Appendix B; Figure 28). StereoInvestigator software (MBF Biosciences, Colchester VT) was used to perform unbiased cell counting within the entire NAc. Estimated total number of cells was calculated using the optical dissector (Gundersen et al. 1988). All stereology was carried out using StereoInvestigator 7 on a Microphot-FXA Microscope (Nikon, Inc, Tokyo Japan) equipped with a MicroFire Megapixel Digital Microscope Camera System (Optronics, Inc, Goleta, CA) and BioPrecision Automated Stage (Ludl Electronic Products, Ltd, Hawthorne, NY).

2.6. ENDOCRINE MEASURES

2.6.1 Brain Collection

Dam brains were dissected from the skull, flash-frozen on dry ice, and stored at -80° C until dissection. Brains were incompletely thawed to allow hand dissection into the regions described using anatomical landmarks in the standard Rat Brain Atlas (see Appendix B, Table 3) (Paxinos & Watson 1997). The amount of time any region was allowed out of -80° C conditions was less than 10 minutes; therefore, none of the tissue was completely thawed at any point before the time of assay.

2.6.2 Plasma Collection

Trunk blood was collected into vials containing 500 KIU/ml of aprotonin and 0.0634M

EDTA (Sigma-Aldrich, St. Louis, MO). Vials were immediately centrifuged at 4°C at 10,000g for 10-15 minutes. Plasma was collected, immediately frozen, and stored at -80°C until further testing.

2.6.3 Blood Collection

Rat dam CORT sample collection was accomplished via a tail nick. Briefly, the dam was wrapped in a cloth towel to provide soft restraint. The collection site was swabbed with betadine (povidone iodine) and alcohol, nicked with a surgical blade, and resulting blood collected into a centrifuge tube containing 0.0634M EDTA. After collection, direct pressure with sterile gauze and elevation was applied to the site until bleeding stopped. Additionally Kwik-Stop® Styptic powder with benzocaine was applied to the site to hasten clotting. Once bleeding had been controlled, the dam was released from towel restraint and allowed to recover in her home cage.

2.6.4 Extraction of Oxytocin Peptide from Plasma

A strata-X 33µm polymeric reversed phase SPE sorbent was equilibrated in a 96-well plate containing 60 mg sorbent per well (Phenomenex, Torrance CA) by adding 1 ml MeOH followed by 1 ml of water. 800 uL of plasma was acidified with .4 ml of 1.5% trifluoroacetic acid (TFA) and centrifuged at 6,000g for 20 minutes at 4°C. This supernatant was loaded onto the pre-treated strata-X plate. Wells were washed with 1.5 ml of 0.1% TFA, and then the peptide eluted with 1 ml of 80% acetonitrile. The eluant was collected in a polystyrene tube, evaporated to dryness under a nitrogen (N2) stream, and the residue reconstituted in 200 ul of assay buffer. Extraction efficiency was determined by spiking one positive control with a known amount of hormone and extracting with the other samples.

2.6.5 Oxytocin Enzymeimmunoassay

OT levels from extracted plasma were measured using an assay kit and protocol from Assay Designs, Inc (Ann Arbor, MI). The endogenous OT hormone competed with OT linked to alkaline phosphatase for the OT antibody binding sites. After the overnight incubation at 4°C, the

excess reagents were washed away and the bound OT phosphatase was incubated with substrate and after 1 hour this enzyme reaction, which generates a yellow color, was stopped. The optical density (OD) was read on a Sunrise plate reader (Tecan, Research Triangle Park, NC) at 405nm. The intensity of the color is inversely proportional to the concentration of OT in the sample. The hormone content (pg/ml) was determined by plotting the OD of each sample against a standard curve. The sensitivity of the assay is 11.6 pg/ml with a standard range of 15-1000 pg/ml. The intra- and inter- assay variation is 4.8% and 8% respectively. Assay Designs reports cross-reactivity for similar neuropeptides found in mammalian sera at less than 0.001.

2.6.6 Corticosterone Radioimmunoassay

Sample CORT measurements were measured using the Corticosterone ^{125}I Radioimmunoassay Kit (MP Biomedicals, Orangburg, NY). Samples were brought to room temperature and steroid diluents, ^{125}I-CORT, and anti-CORT were incubated for 2 hours. Precipitant solution was added, vortexed, and centrifuged at 1000g for 15 minutes. The radioactivity in the pellet was measured using a LKB CliniGamma counter, which calculates the nanogram content of CORT in each sample from the standard curve. The intra-assay and inter-assay coefficients of variance were 4.4% and 6.5%, respectively.

2.6.7 Oxytocin Radioimmunoassay

For brain regions, tissue was processed as previously described (Johns et al. 1997). Briefly, tissue was homogenized in buffer and centrifuged. OT immunoreactive content was assayed in the supernatant according to a protocol from Bachem/Peninsula Labs (Belmont, CA). Samples and standards (1.0 – 128.0 pg) were incubated in duplicate with anti-OT serum. This was followed by incubation with ^{125}I-OT after which time normal rabbit serum and goat anti-rabbit IgG serum were added. The ^{125}I-OT bound to the antibody complex was separated by centrifugation. The radioactivity in the pellet was measured using a LKB CliniGamma counter (PerkinElmer Wizard 1470-005), which calculates the picogram content of OT in each sample

from the standard curve.

CHAPTER 3. GESTATIONAL COCAINE EXPOSURE: EFFECTS ON RETRIEVAL PREFERENCE AND PUP-DIRECTED BEHAVIORS

3.1. INTRODUCTION

Rodents exhibit stereotyped behaviors toward infant rat pups that can be easily quantified and these activities can be divided into appetitive (goal-directed behaviors, such as pup retrieval) and consummatory (behaviors once the goal has been obtained, such as nursing). Bouts of licking and grooming, another pup-directed behavior, may have both appetitive (approaching and handling the pup) and consummatory (licking and grooming sequences) aspects (Numan & Stolzenberg 2009). Dam retrieval of pups changes over repeated testing (consequently across the postpartum period); however, whether this is caused by maternal experience, hormonal changes, or dynamic changes in pup cues (auditory or olfactory) is unknown (Hahn & Lavooy 2005).

Gestational cocaine exposure is known to cause a variety of deficits in MB, including specifically an increased latency to retrieve pups back to the nest (Johns et al. 1997). This may indicate that CC-treated dams lack maternal motivation or are less sensitive to the pup-produced cues that allow pups to be located outside of the nest. Additionally, CC-exposed pups are less able to elicit MB from both UN and CC-treated dams (Johns et al. 2005), suggesting there may be some aspects of CC-treated pups that are aversive. This previous study focused on maternal responses to cross-fostered pup litters of one type (i.e. dams were given either UN or CC-treated pups), but did not study choice behavior of dams between the different types of pups.

The current study was therefore designed to determine if a. CC treatment affects maternal retrieval response and pup choice, independent of the effects of pup CC exposure; and b. CC-exposed pups elicit less MB from all dams compared to non-exposed pups

3.2. METHODS

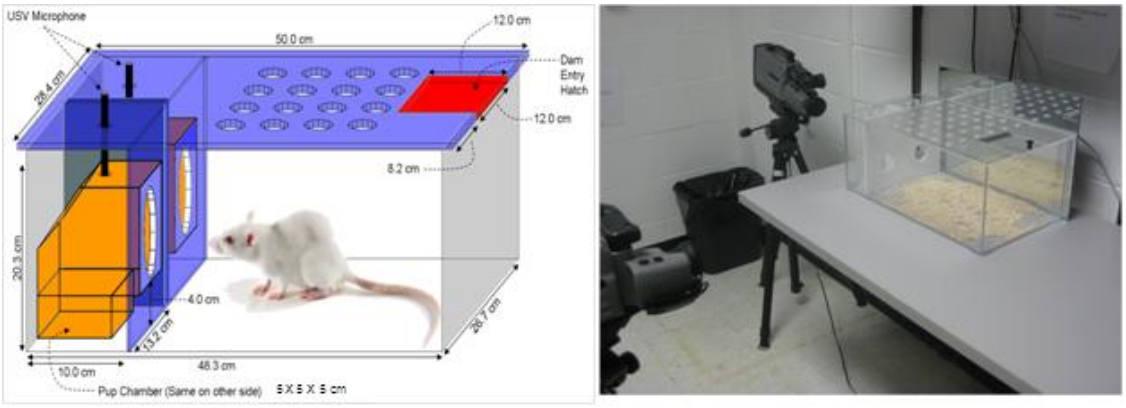

Figure 5. Retrieval Testing Apparatus.

On left: diagram of chamber with dimensions. On Right: experimental set-up with cameras. Two pup chambers (yellow) large enough to hold 4 pups only. Each chamber would hold opposite treatment condition pups (i.e. UN pups vs CC-treated pups). The rest of the chamber was large enough for dams to establish a nest area, but also be able to rest away from pups. Fresh litter lined the floor for every test. Two cameras were used to videotape each session. USV: ultrasonic vocalization

3.2.1 Apparatus

The apparatus (Figure 5) consisted of a plexiglas cage (27 cm X 50 cm), a ventilated plexiglas top with a door, and two pup chambers, each large enough to hold four pups and from which the dam could extract pups. Dams were unable to completely enter the pup chamber. Above each pup chamber is an extension cone directing the recording field of the attached USV detector towards the interior of the cage. To begin the test, dams were lowered into the retrieval chamber via the hinged door. Pilot experiments indicated that both UN and CC-treated dam behavior was similar in this apparatus compared to home cage testing (see Appendix C; Table 4). This apparatus design ensured that remaining olfactory cues from the home cage could not interfere with retrieval preference behavior. Fresh litter was added before every session, and the apparatus was cleaned with a non-toxic spray (Greenworks All-purpose Cleaner, Chlorox®

Oakland, CA) after every animal. Urination and defecation are autonomic responses that can occur when the animal is anxious or fearful (Antoniadis & McDonald 2001); therefore to assess whether the retrieval apparatus induced any emotional differences in the animals these measures were recorded. There were no differences due to treatment on urination or defecation in the chamber on any day (GDs 18/19, PPDs 1/3/5). These results gave us confidence that the retrieval chamber did not induce disproportionate fear responses in any one treatment group.

3.2.2 Habituation to Apparatus

On GDs 16 and 17, dams were habituated to traveling to the test room. They were transported in their home cages to the testing room, allowed to acclimate for ten minutes, and then returned to the colony room. On GD 18, dams were brought to a procedure room, placed in a fresh cage and taken to the testing room. Dams were placed in the chamber for ten minutes. Pilot testing indicated this duration resulted in a greater than 90% of dams resting by the end of the session. Dams were given 4 pieces of cereal to find in each pup chamber, to test their ability to smell and consume the cereal and habituate to investigating the pup chambers. Retrieval of cereal was recorded. UN dams were less likely to retrieve fruit loops compared to CC-treated and CS-treated dams (χ^2 (2) = 3.087, $p < 0.05$) see Appendix C Table 5), suggesting that gestational treatment paradigms did not cause deficits in olfactory ability. Following the test, dams were returned to their home cages. In addition to retrieval box testing, rats were habituated to an olfactory chamber (See Chapter 5). These habituations occurred in series. The order of habituations was cross balanced for order and time of testing. On GD 19, the same habituation procedures were performed except cereal was not introduced into the retrieval chamber. Autonomic responses (urination and defecation) were recorded at each session indicated that all groups habituated (i.e. fewer autonomic responses) on GD 19 compared to GD 18 (χ^2 (1) = 4.098; $p \leq 0.05$).

3.2.3 Retrieval Testing Procedure

Test dams and pup-providing dams were brought into the procedure room. Pups used in the retrieval test (two of each sex, four of each treatment) were marked three times on their heads, stomachs, and backs with Sharpie markers in order to make recognition of pup retrieval order easier (See Figure 6). Pups from the test dam were also marked to habituate the dam to the smell of the marker. This marking did not affect retrieval or licking behavior in the home cage. After 20 minutes of room habituation the dam's preference for olfactory stimuli was tested (see Chapter 5). These procedures resulted in a 30 minute total pup separation time similar to separation times in previous MB studies with CC-treated, CS-treated and UN rat dams (Johns et al. 1998, Johns et al. 1994, Johns et al. 2005).

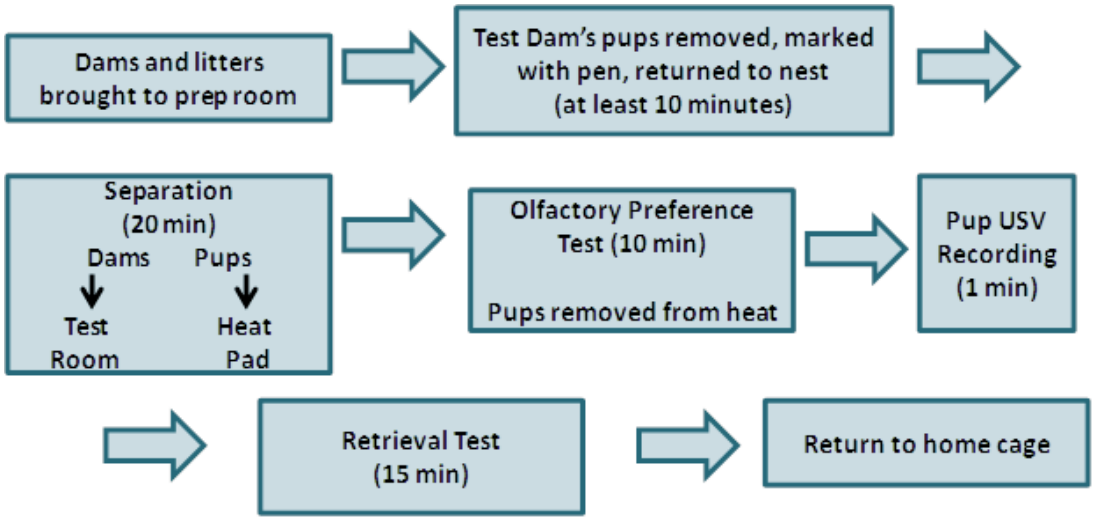

Figure 6. Experimental Procedure For Retrieval Testing.

The entire testing protocol lasted approximately 1.5 hours. Test dams were used for olfactory preference testing prior to retrieval. Dams were sacrificed for endocrine testing on PND 5 (see Chapter 5). USV: ultrasonic vocalization

Following USV recording (see Chapter 6), the dam was placed into the box and the timer was set for fifteen minutes. Two VHS low light sensitivity video cameras were used: one to monitor the pups' behavior in the chamber and one to monitor the dams' behavior. An observer stayed in the room to record the order according to sex and prenatal treatment condition in which

the dam retrieved the pups. After all of the pups were retrieved, the observer left the room for the remainder of the test.

3.2.4 Experimental Design

Experiment 1: Pup Prenatal Exposure Preference Across Days

Gestational treatment is described in Section 2.2 and 2.3. Pup provider pups were used for both treatment groups on PPDs 1 and 3, but on PPD 5, dams had a choice of either their own pups or pups from the opposite treatment of a pup provider litter (i.e. CC-treated dam choose between own CC-exposed pups and surrogate UN pups; See Figure 7).

Surrogate Pups	Postpartum day					
Experimental Dam Pups	1		3		5	
Dam treatment	Maternal Retrieval and Behavior		Maternal Retrieval and Behavior		Maternal Retrieval and Behavior	
CC (n=11-13)	CC pups	UN pups	CC pups	UN pups	CC pups	UN pups
CS (n=13-15)	CC pups	UN pups	CC pups	UN pups	CC pups	CS pups
UN (n=11-13)	CC pups	UN pups	CC pups	UN pups	CC pups	UN pups

Figure 7. Experimental Design Across Test Days For Retrieval Testing.

On PPDs 1 and 3, experimental dams (red boxes) chose between surrogate UN or CC-exposed pups (blue boxes) from pup-provider dams. On PPD 5, experimental dams chose between their own biological pups CC-exposed, CS-exposed, or UN pups (red boxes) and surrogate pups.

Experiment 2: Own Pup vs Other Same Treatment Pups

On PND 5, a separate set of UN and CC-treated dams were tested for preference for their own pups versus unfamiliar pups from the same treatment condition (i.e. UN-own vs. UN-other). All other experimental designs were maintained.

3.2.5 Analysis of Retrieval Preference Behavior

Behavioral Coding: See General Methods for Procedural Details of Coding Software. Behaviors coded included: “Retrieve One Right” or “Retrieve One Left” each time the dam retrieved one pup from the respective chamber. “Retrieve All Right” or “Retrieve All Left” coded when the dam retrieved the fourth (last) pup from a certain side. “Head In” coded when the dam

placed her entire head (past both ears) into the retrieval chamber. After all pups on a side were retrieved, "Head In" behavior was coded as "Other" behavior. "Touch" coded every time the dam was in contact with the pups, including when the dam was sitting on top of the pups, except when she was self-grooming (scored as "Other") or licking the pups. "Lick" was coded when the dam licked the pups with her tongue. If unable to determine if the dam was touching or licking based on her position in the test box, the behavior was recorded as "Touch." "Crouch" was coded when the dam was in a high dorsal arched-back position with the pups underneath her. This is a sustained position, so the dam had to hold the position for at least five seconds before it was coded as a crouch. "Group" was coded when single pups were placed together near other pups. "Other" was scored for behaviors aside from the behaviors previously mentioned, including self-grooming, circling, nest-building, searching for pups in empty chambers, or chamber exploration.

3.2.6 Statistical Analysis

Two-way analysis of variance (ANOVAs; dam treatment X pup treatment) were run for latency measures of retrieval behaviors with post-hoc Bonferonni tests performed to compare specific group differences when main or interactions effects reached significance (alpha = $p \leq 0.05$). One-way ANOVAs (dam treatment) were run for frequency and duration measures of non-retrieval MBs, with post-hoc Bonferonni tests performed to compare specific group differences (alpha = $p \leq 0.05$). Chi square tests were used to determine if there were differences in the proportion of dams exhibiting specific behaviors.

3.3. RESULTS

3.3.1 Experiment 1: Chronic Cocaine versus Untreated/Chronic Saline Pups

Retrieval Latency

Two-way ANOVA revealed that CS-treated dams retrieved all 8 pups sooner on PND 1 than did UN dams (F (1,24)= 6.147, $p \leq 0.05$) and CC-treated dams (F (1,24)= 6.897, $p \leq 0.05$),

regardless of pup treatment (see Appendix C; Table 6). There were no significant differences in latency to retrieve on PNDs 3 or 5. Additional analyses indicated that there were no differences in test day, litter treatment, or dam treatment on the interval between retrieving the first pup, the first litter, or all 8 pups.

Maternal Behaviors

There were a number of effects in non-retrieval MBs across treatment groups and test days. On PPD 1, CS-treated dams touched pups for longer than UN (F (1,31) = 10.675, $p \leq 0.005$) and CC-treated dams (F (1,31) = 6.575, $p \leq 0.01$). However, CC-treated dams touched pups more often compared to CS-treated dams (F (1,30) = 5.342, $p \leq 0.05$), and a trend was observed to have touched more than UN dams (F (1,30) = 4.041, $p \leq 0.055$). CS-treated dams exhibited fewer non-pup directed behaviors compared to UN (F (1,29) = 19.275, $p \leq 0.001$) and CC-treated dams (F (1,29) = 7.076, $p \leq 0.05$). On PPD 3 UN dams touched pups more often than CC-treated dams (F (1,35) = 7.073, $p \leq 0.05$) and CS-treated dams (F (1,35) = 7.232, $p \leq 0.05$). On PPD 5 UN dams touched pups for a longer duration compared to CC-treated (F (1,36) = 10.779, $p \leq 0.005$) and CS-treated (F (1,36) = 6.037, $p \leq 0.05$) dams. Repeated measures analysis also indicated a main effect of age across all groups such that licking frequency (F (2,56) = 4.383, $p \leq 0.05$) and touching duration (F (2,56) = 16.121, $p \leq 0.001$) were decreased, while non-pup-directed behaviors increased in duration (F (2,56) = 33.654, $p \leq 0.001$) and frequency (F (2,56) = 3.495, $p \leq 0.05$). See Table 7 in Appendix C for data.

Pup Gender Effects

We investigated the proportion of dams that retrieved a male pup first or retrieved all males before all females. Preference based on gender differed across days (See Figure 8 A, B.) CS-treated dams were more likely to retrieve a male compared to UN dams on PPD 3 ($\chi 2(1) = 5.239$, $p \leq 0.05$).

Litter Treatment Effects

Since there was a great deal of variability in the behavior of dams across days and within treatment groups, we assessed the proportion of dams in each group that exhibited specific characteristics of retrieval behavior, including the proportion of dams retrieving an UN pup first or all 4 UN pups first. Dams differed slightly across all the test days (See Figure 8 C, D). UN dams were more likely to retrieve a UN pup first on PPD 3 compared to CS-treated dams ($\chi 2$ (1) = 3.896, $p \leq 0.05$) and a trend toward significance was observed compared to CC-treated dams ($\chi 2(1) = 3.143$, $p \leq 0.08$). CC-treated dams exhibited a preference to retrieve a CC-exposed pup first and CC-exposed litter first on PPD 5 but this distribution was not statistically significantly different. Preference for a litter was not related to preference for the first pup, and no significant differences in preference choice across groups.

3.3.2 Experiment 2: Own versus Other same Treatment pups

Given the strong preference exhibited for their own CC-exposed pups over unfamiliar UN pups on PPD 5, we tested a separate group of UN and CC-treated dams on PND 5 to determine if the preference for own pups was based on biological status or prenatal drug treatment. CC-treated dams showed no clear preference between their own pups and other CC-exposed pups (Figure 8 C,D), while UN dams always retrieved their own biological pups before unfamiliar UN pups. CS-treated dams were not tested since they did not show a similarly strong preference for their own pups and they were not hypothesized to be different from UN dams on this measure.

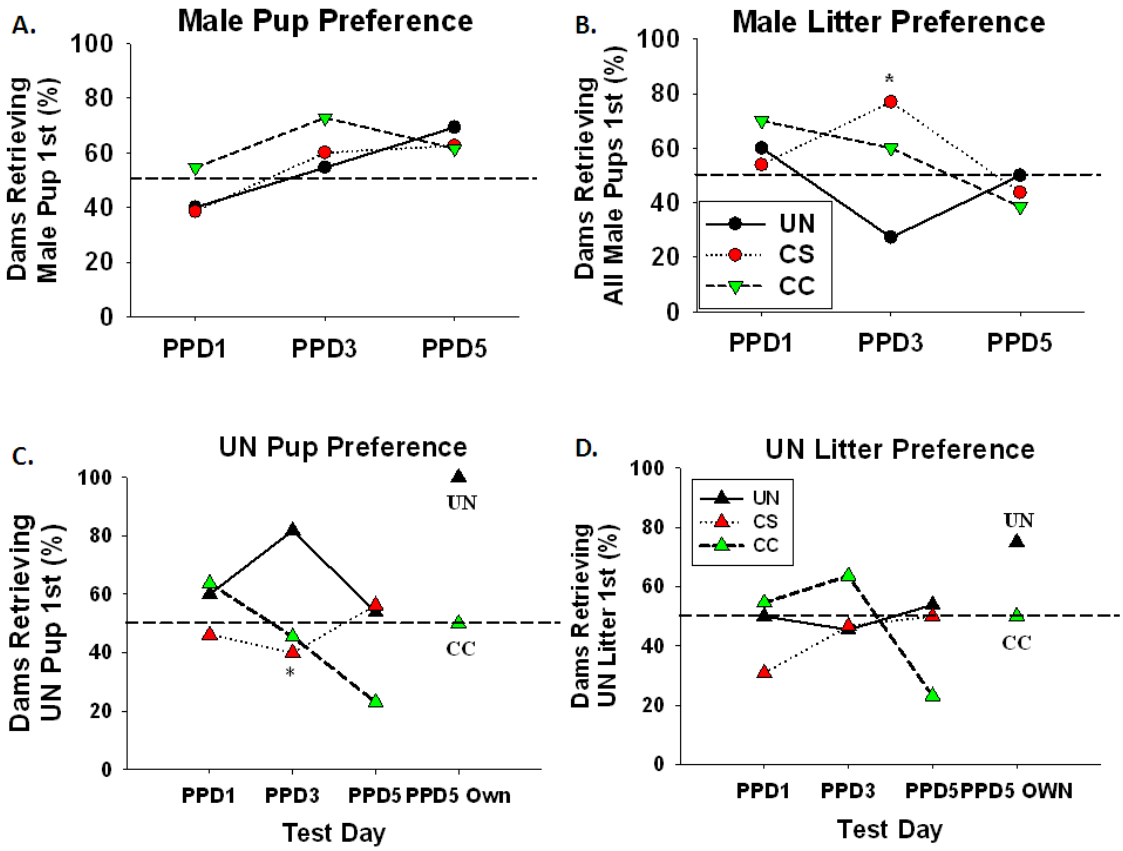

Figure 8 Maternal Retrieval Preference Behavior.

All data are presented as percentage of each treatment group to exhibit a specific preference. Preference was defined as which pup(s) were retrieved first. A., C.) Preference for the first pup to be retrieved. B., D.) Preference for the first litter (4 pups) to be retrieved. A.,B.) Results of preference for pup sex. C., D.) Results of preference for pup prenatal condition. Line graphs indicate groups that were repeatedly tested. Single points represent separate test dams for 'Own vs Other' comparisons. Asterisks (*= p≤ 0.05) indicate differences from UN dam behavior.

3.4. DISCUSSION

Our hypothesis that CC-treated dams would exhibit greater latency to retrieve pups across test days was not supported; although previous studies have shown that CC treatment increases retrieval latency on PPD1 (Johns et al. 1997). The lack of effect may be caused by the immense variability observed in retrieval latency across groups and test days. However, our results suggest that retrieval preference behavior differs interactively dependent on dam treatment, pup treatment and postnatal day tested. CC-treated dams showed a different pattern of retrieval behavior across

the test days compared to UN and CS-treated dams. They did not show a strong preference for the initial pup chosen or initial litters retrieved on PPD 1, but were slightly more likely to prefer retrieving UN litters by PPD 3 and to retrieve their own CC-exposed pups first on PPD 5. Although the proportion of CC-treated dams did not differ significantly from UN and CS-treated dams, we believe that a larger sample size may confirm a difference in proportions, especially on PPD 5. If confirmed, it would suggest that CC-treated dams either develop a preference for CC-exposed pup cues over the first postpartum week or, alternatively, prefer familiar pups to unfamiliar pups regardless of treatment exposure by PPD 5. Similar to previous experiments (Johns et al. 2005, Johns et al. 1997, Johns et al. 1994, Nelson et al. 1998), we observed that CC-treated dams spent more time engaging in non-pup-directed behavior by PPD 5 compared to UN dams, suggesting CC-treated dams may have become accustomed to the novelty of the retrieval task and have reverted to home-cage typical behaviors (less MB).

Interestingly, we observed that CS-treated dams also differed from UN dams in retrieval and MBs. CS-treated dams retrieved all pups faster than UN and CC-treated dams on PPD 1, likely explaining the differences in touching between dam treatment groups on that day. CS-treated dams did not show a clear preference for pup type at any test day, with the exception of preferring male litters on PPD 3, suggesting that they do not differentiate between CC-exposed and control (UN/CS-exposed) pups. CS-treated dams also show reduced pup contact by PPD 5 compared to UN dams, appearing more similar to CC-treated dams in their behavior. This would suggest that the gestational treatment affects CS-treated dam behaviors but that differences from UN dams are not observed until the task becomes more complicated or stressful, as CS-treated dams do not differ from UN dams in previous studies (Johns et al. 2005).

Pup prenatal exposure condition did not influence litter retrieval preference or latency on any day in UN dams, although a preference for initially retrieving UN pups on PPD 3 was observed. UN dams may have displayed the more typical behavior of early postpartum rat dams to retrieve and care for most pups within the first few days of delivery. As they also spent more

time in contact with pups on PPD 5 compared to previous days, they may also have habituated to the retrieval task; or perhaps, as some of the pups were hers on PPD 5, dams were more motivated to stay with them. Experiment two revealed that UN dams preferred their own pups compared to unfamiliar UN pups, similar to what has been found in other studies (D'Amato et al. 2005), however a lack of a clear preference for UN pups compared to CC-exposed pups on PPD 5 was observed in experiment one.

Additionally, comparisons of preference behavior of UN and CC-treated dams between experiments 1 and 2 on PPD 5 suggest that some pup-produced cues may be different between UN and CC-exposed pups (see Chapter 6). In the preliminary data presented from experiment 2, when given the choice between litters of similar prenatal treatment conditions UN dams are more likely to retrieve their own pups, while CC-treated dams do not exhibit a clear preference for biological pups. Compared to experiment 1, this is large difference in the likelihood to choose their own pups; UN dams become less likely to retrieve their own pups and CC-treated dams become more likely to prefer their own CC-exposed pups, indicating that it is not novelty per se, but instead, the differences between CC-exposed and UN pups that is important to dams. These data suggest that some aspect of CC-exposed pups was capable of attracting more attention or was perceived as more attractive. A similar effect was observed in PC-exposed human infants which elicited more attention from care-givers in a laboratory session (Lewis et al. 2009). However, this initial attention does not guarantee sustained attention, therefore it may not explain differences in other pup-directed behaviors observed previously (Johns et al. 2005) Potential mechanisms, underlying behavioral differences are most likely to be olfactory and auditory cues (see Chapter 6).

Given that retrieval behavior specifically has been tied to USVs, it is possible that CC-exposed pups are emitting USVs with different spectral characteristics, although several USVs aspects do not differ (see Chapter 6 for USV data). It is unlikely that CC-treated dams have perceptual auditory deficits since it not alter sensorimotor gating (see Appendix C; Figure 29).

However, plasticity within the auditory cortex itself occurs during pregnancy and lactation, and it has recently been suggested that this plasticity depends on not only hormonal changes, including OT, but also DA, serotonin (5-HT) and norepinephrine (NE) signaling, all of which can be affected by chronic cocaine use. Alternatively, CC-treated dams may be more or less sensitive to olfactory cues from pups (see Chapter 5). Furthermore, retrieval behavior is believed to be an appetitive behavior, dependent on MPOA activation of the VTA (Numan & Stolzenberg 2009). This response involves OT in several brain regions and may involve peripheral OT as well. Given that CC treatment reduced OT in other studies (Johns et al. 1997), this potential mechanism is explored in Chapter 4.

One major limitation in the interpretation of results of retrieval preference behavior is the great degree of variability in behavior within treatment groups and test days. This task was performed in a novel apparatus with no established 'nest' area in the testing cage, which meant that there was no initial safe place to retrieve pups. Although, once a 'nesting' area was established most dams would group pups together. This variability may be reduced if the task were simpler such as home-cage choice task or choice for a single pup of each treatment. Such studies are currently ongoing in the lab to address these questions; but, while this may be a limitation for comparative purposes, this was meant to be a task involving a requirement for more "motivated" behavior as dams had to find and pull pups from an internal compartment. That all dams did retrieve pups from the chambers is important as it meant pup cues were perceived and elicited a reaction. However, to determine motivational saliency of pups, conditioned place preference or greater obstacles to retrieval could be tested between CC-treated dams and the control groups.

There was little consistency across time in dam behavior but that may also have been normal, in the sense that dam response would vary over time as experience and pup cues change. Additionally, there were clear differences in retrieval preference for pup sex, however, we did not have the statistical power to look for differences in response to and interaction of pup treatment

and pup sex (i.e. CC-exposed males vs CC-exposed females). This is an intriguing question as PC-exposure can have sex-specific effects on offspring, but could have contributed to the variability in pup cues, thus increasing variability in response to the pups.

Taken together, these data suggest that dams' initial response to pup choice may not be indicative of the later MBs toward pups from specific prenatal treatment conditions (CC-exposed vs UN). Future studies may study the more intricate details of maternal-infant interactions over longer intervals of time and across the postpartum period to more fully determine mechanisms of cocaine-induced maternal neglect.

CHAPTER 4. GESTATIONAL COCAINE EXPOSURE DISRUPTS OXYTOCIN SIGNALING AND STRESS RESPONSE IN RAT DAMS

4.1. INTRODUCTION

The role of the endocrine system in regulating maternal physiology and behavior is well established, with hormones such as estrogen, progesterone, prolactin, CORT, and OT shown to be important signals to organize physiology and neurobiology for a smoother transition between pregnancy and lactation (i.e. nulliparous to primiparous transition) (Weiss 2000, Brunton & Russell 2008). The gestational, peripartum and postpartum periods are typically characterized by high basal blood CORT levels, and a hyporesponsivness to stress evidenced by blunted CORT responses to physiological and psychological stressors (Neumann 2001, Shanks & Lightman 2001, Windle et al. 1997, Carter et al. 2001, Slattery & Neumann 2008). It would appear that maintaining stress hormones within a strict range is required for optimal MB as perturbances in either direction can disrupt MB. Administering CORT to rats either during pregnancy, the postpartum period, or both decreases nursing and increases neglectful behaviors (Brummelte & Galea 2010). Conversely, removing circulating stress hormones can also reduce but not abolish MB in the early postpartum (Rees et al. 2004). Peripheral OT (measured in plasma) is well known for its role in uterine contractions and milk ejection (Lee et al. 2009). It remains unclear whether plasma OT levels are correlated in a meaningful way with central OT in other brain regions critical for MB in animal models, but such a relationship would be important as plasma OT is currently one of the main measures of OT used in human studies.

Plasma OT has been described as an ‘anti-stress’ system with plasma levels correlated with specific hypothalamic OT levels during stress (Engelmann et al. 2006), along with its interaction

with the regulation of CORT, (Carter et al. 2001, Slattery & Neumann 2008, Coppens et al. 2010). Chronic OT is known to reduce acute stress response (Windle et al. 2004) and lactation, presumably through high OT levels, can attenuate response to stress in the parvocellular cells of the PVN (Da Costa et al. 2001). Disruption of the interactions between OT and CORT in the postpartum period may lead to differential levels of anxiety or depressive-like behavior which could underlie differences in MB. There is an established bidirectional relationship between substance abuse and stress-related symptomatology (Sinha 2001, Goeders 2002, Koob & Volkow 2010). Cocaine acutely activates the HPA axis (Goeders 2002), a response that is upregulated by female sex hormones. HPA reactivity is heightened during acute withdrawal and dysregulation persists during protracted abstinence (Goeders 2002, Corominas et al. 2010). Importantly, CC can raise CORT levels significantly during pregnancy (Quinones-Jenab et al. 2000), although the impact on feedback regulation is less clear. Cocaine-using women exhibit disruptions in CORT signaling as well as reduced plasma levels of OT both at baseline and following an infant-interaction session compared to control moms (Light et al. 2004).

Cocaine use, depression and anxiety are highly correlated in clinical populations (Rounsaville 2004, Hans 1999), specifically in postpartum women (Singer et al. 1995, Hans 1999). Mood disorders can negatively impact maternal-infant interactions independently of drug use (Smith et al. 2004, Braw et al. 2008, Leahy-Warren & McCarthy 2007, Noorlander et al. 2008), and they may contribute to disrupted maternal-infant interactions in drug-abusing women. Stress during pregnancy can reduce MB in rodents, however, if the rats were prone to having low MB, stress did not affect them, suggesting that optimal care can be reduced but not minimal (Champagne & Meaney 2006). Both acute and repeated stressors during mid-gestation reduced pup-directed behaviors on PPDs 2, 4, and 6 in rat dams (Patin et al. 2002). Repeated stressors during the postpartum can inhibit lactation in rodents, suggesting direct hormonal effects (Lau & Simpson 2004).

Stress-induced deficits in MB may be caused by changes in central levels of OT. Central

nervous system levels of OT have been extensively studied regarding the important role in promoting MB, and social behavior generally, in both humans and animal models (Lee et al. 2009). The anterior hypothalamus contains several regions important for MB. Along with the paraventricular nucleus (PVN) and supraoptic nucleus (SON) which produce OT, the MPOA, and ventral BNST which are critical for the initiation and maintenance of MB reside within the anterior hypothalamus (Numan & Stolzenberg 2009). The MPOA has been implicated in the appetitive facets of MB mediating activity in both the reward and stress circuitries during the postpartum period (Numan & Stolzenberg 2009). CC treatment in rodents decreases OT levels in several brain regions, including the MPOA, HIPP and VTA (Johns et al. 1997) during the early postpartum period when MB was disrupted. It remains unclear if cocaine treatment also affects circulating OT levels in the postpartum rat at critical points during MB expression.

Given 1) the importance of OT signaling (both centrally and peripherally) in MB and stress, 2) stress and affective disorders can underlie deficits in MB, and 3) cocaine has been shown to disrupt both MB and stress independently, we proposed several studies to investigate the relationships between these factors in the postpartum period. These studies were designed to determine: a. if maternal response to pups is correlated with peripheral and central OT level differences in CC-treated versus control (CS-treated, UN) dams; b. if CC-treated dams differ in measures of anxiety, stress or depressive like behavior in the postpartum period; c. if HPA axis function (through OT and CORT measures) differs in CC-treated dams compared to UN dams following behavioral testing; d. if plasma OT and specific brain region OT levels correlate in postpartum dams dependent on treatment.

CC-treated, CS-treated and UN rat dams were tested for hormone levels following maternal retrieval on PPD 5 (see Chapter 3). Plasma OT and CORT levels and dam brains were extracted for OT level assessment in specific regions associated with maternal and social behavioral response. My hypotheses were that: a. CC-treated dams would have lower plasma and brain region OT levels than control dams; b. CC-treated rat dams would exhibit higher anxiety and

depressive-like behavior on PPD 5 compared to UN dams correlated with higher baseline and post-behavior CORT blood levels; c. OT levels in the brain (hypothalamus, VTA, and AMY) would correlate with OT plasma levels (i.e. high plasma level, high brain region level).

4.2. EXPERIMENTAL DESIGN AND METHODS

4.2.1 Experiment 1: Dam Endocrine Measures Following the Retrieval Test

Breeding and Gestational Treatment Procedures can be found in Chapter 2 (General Methods). The tissue used for these experiments was collected from the animals used in retrieval preference testing (Chapter 3). Following the retrieval preference testing on PPD 5, crouching status was noted and dams were returned to the rest cage until time of sacrifice. The time between the end of the retrieval test and sacrifice was recorded and was never greater than 10 minutes. Dams were decapitated and trunk blood and brains were immediately collected (see Chapter 2-Methods for specific details). Brains were dissected and the NAc, anterior hypothalamus (MPOA/PVN/SON), AMY, and VTA were collected (see Table 3 in Appendix B).

Statistical Analysis

Following removal of outliers (greater than 2 standard deviations from the mean), 1-way ANOVAs were performed on OT and CORT plasma levels. One-way ANOVAs were run for OT levels in each brain region to compare between treatment groups. Linear regression analyses were performed to assess relationships between plasma OT-behavior, plasma OT-plasma CORT, plasma OT-regional brain OT, and between region-OT levels.

4.2.2 Experiment 2: Stress, Anxiety and Endocrine Response

Two-five days prior to mating, all females were placed in the Open Field Test (OFT) apparatus. Data from this day was used as a ‘baseline’ anxiety measure to account for the natural variation that can occur within large groups of animals (Coppens et al. 2010). Animals were delivered to the facility in ‘cohorts’ for testing. All members of a cohort underwent OFT within 2

days of each other and following 5-10 days of habituation to the animal facility with 2 days of travel and room habituation. On PPDs 1, 3 and 5, test dams for these tasks were brought to room for approximately 30 minutes and pups from each dam were removed (for USV and urine collection) while the dam remained in the home cage for approximately 30 minutes. On the morning of PPD 5, two hours after pups were returned (from retrieval tests see Chapter 3), CC-treated and UN dams were randomly divided into 3 test groups or 'types'. See Figure 9 for an overall schematic of the behavioral and hormonal testing procedures. CS-treated dams were not used in these studies since their behavior did not differ from UN dams on PPD 5 retrieval testing.

Type 1 dams were tested for behavior and hormonal levels. Tail blood was collected (see Chapter 2-Methods) and dams were returned to their home cage in the test room with pups present for two hours. The dams were then placed in the OFT chamber for 10 minutes and five minutes following the conclusion of the OFT, tail blood was again drawn, and the dam placed back in her home cage for a second 2 hour rest period. Dams were then placed in the forced swim test (FST) tank for a 10-minute test after which (five minutes) they were sacrificed for blood and brain collection.

Tail blood collection has been shown to increase stress hormone levels if the procedure takes longer than 3 minutes (Vahl et al. 2005) and high stress hormones can affect anxiety-like behavior (Holsboer & Ising 2008). Therefore, Type 2 control dams were tested for behavior on a similar time schedule, but tail blood was not collected. CORT exhibits a diurnal rhythm of release that is maintained in lactating rats (Atkinson & Waddell 1995), however this may be affected by CC treatment. Therefore, Type 3 control dams were used to measure how blood hormone levels may change throughout the day without having performed the behavioral tasks.

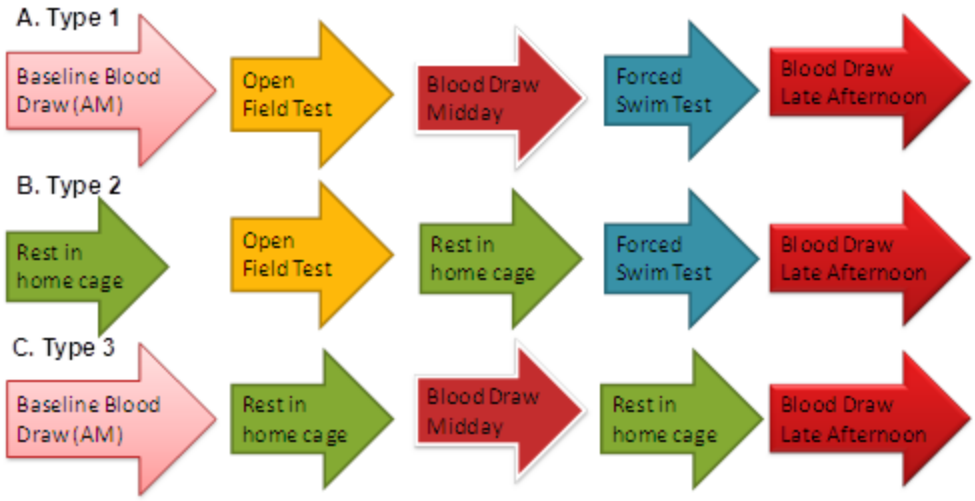

Figure 9. Experimental Design for Stress and Endocrine Testing.

UN and CC dams were divided into 3 'Types' based on P5 schedule. Blood draws taken for baseline measurements (pink arrows) were collected in the morning. Later blood draws (red arrows) were taken after rest (green arrows), following open field testing (yellow arrows), or forced swim testing (blue arrows). All dams were sacrificed at the last blood draw.

It was observed that blood draw procedure could increase center time in the OFT in Type 1 dams compared to Type 2 dams (see Appendix D; Figure 30). There was no difference between the treatment groups on this measure so Type 1 and Type 2 data were pooled for final analyses.

All dams were sacrificed and trunk blood and brains were collected at the end of testing (See Chapter 2: General Methods). Dams had anterior hypothalamus and AMY dissected out for OT analysis (see Table 3 in Appendix B.).

Statistical Analysis

One-way ANOVAs were used to evaluate differences among cohorts in baseline OFT behavior; further post-hoc pairwise comparisons with Bonferonni correction were performed with selected pairwise comparisons adjusted for multiple comparisons type I error rate. Linear models adjusting for baseline measurements were used to explore differences in OFT behavior between treatment groups. For all postpartum OFT tests, we compared UN and CC treatment groups. We also compared the postpartum behavior to the baseline behaviors for each animal to investigate

differences across time, and if CC treatment affected the progression of anxiety-like behavior through the early postpartum period.

Two-way ANOVAs (Type x Treatment) were used to assess behavioral differences in FST behavior. To determine differences between CORT and OT plasma or brain levels, two-way ANOVAs (Treatment X Type) or Wilcoxon signed rank test were used given the non-normality of the CORT data. Linear regression analyses were performed to assess relationships between plasma OT-behavior, plasma OT-plasma CORT, plasma OT-regional brain OT, and between region-OT levels.

Open Field Testing (OFT)

The open field chamber (61 cm by 64 cm) had dark opaque flooring and walls (38 cm high). Subjects were brought to the testing room and allowed to habituate for at least 30 minutes prior to the test. Animals were removed from the home cage and placed in the OFT chamber for 10 minutes and allowed to explore freely, while the experimenters left the room. Following the test animals were placed back in the home cage and any urination or defecation was noted. The OFT chamber was cleaned with a non-toxic spray (Greenworks All-purpose Cleaner, Chlorox® Oakland, CA) after each test. All tests were videotaped for later analysis. The camera was set up directly above the OFT chamber. It was noted from studies (see Figure 11), that center duration was quite low, however this seems to be a habituation effect (see Appendix D. Figure 30).

Video Analysis for OFT

For videotaping procedures see Chapter 2. The OFT chamber was divided into Wall and Center compartments. The Wall was defined as the area between the outer edges of the chamber up to 10 cm into the chamber. The Center comprised of the rest of the chamber (54 cm X 51 cm). This resulted in the center compartment being approximately 70% of the total space in the chamber. Wall compartment dimensions were chosen based on pilot work determining that this space was typical for female rats to use prior to changing direction along one side of the chamber.

Behavioral coding was performed using Noldus Ethovision Version 3.0 software (Noldus Information Technology, Inc. Version Wageningen, The Netherlands). The animal's location within the chamber was tracked and frequency of entries, duration, total distance traveled and velocity of movement in each chamber was recorded in one-minute bins. Ethovision recorded data in one-minute bins that were then summed (duration, frequency and distance) or averaged (velocity) across the 10 minute session.

Forced Swim Test (FST)

Dams were allowed to rest with pups in the test room prior to the FST. The FST tank (41cm high; 11 cm radius) was filled with tap water (average temperature 22-25° C) to a depth that the rats could not reach the bottom and could not escape the tank. Dams were removed from the home cage; nursing was noted, and placed into the tank for 10 minutes. The dams were then removed from the tank, towel dried and returned to the home cage.

Video Analysis for FST

All test sessions were videotaped (see General Methods-Chapter 2). The behaviors of interest were "Dive": dam put entire head under water and swam to the bottom of the tank; "Climb": dam pressed rapidly alternating forepaws against the wall of the tank above the water line. The body needed to be vertical with the ventrum against the wall of the tank. Swimming was coded depending on the number of limbs moving, thus there was "Swim – two legs"; "Swim – three legs", and "Swim -- 4 legs"; and "Immobile": Animal had no more than one leg moving (typically a back leg in a slow flapping). Tail movements were consistent across behaviors and thus not considered in coding. No swim-three behavior was noted in any test.

For standard blood collection and endocrine assay see Chapter 2-Methods.

For standard brain collection and OT assay see Chapter 2-Methods

4.3. RESULTS

4.3.1 Experiment 1- Dam Endocrine Measures Following Retrieval Testing

A one-way ANOVA revealed that CC-treated dams had lower plasma OT levels following the retrieval preference testing compared to UN and CS-treated dams (F (2,20) = 4.869, $p \leq 0.05$; Figure 10). There were no differences in plasma CORT levels or in relationships between OT and CORT levels (see Appendix D; Figure 32). Linear regression analyses indicated that there was no relationship between plasma OT levels and latency to retrieve the first pup, the first litter or all pups. Approximately 50% of dams from all groups were found to be in the nursing position at the end of the retrieval test, although there was no association with nursing and plasma OT levels. OT levels did not differ between treatment groups in the hypothalamus, NAc, AMY, or VTA (Appendix D; Figure 33). CS-treated dams show a relationship between hypothalamic and NAc OT levels (F (1,11) = 9.098 $p \leq 0.05$). CC-treated dams show a strong relationship between VTA and NAc OT levels (F (1,8) = 5.762, $p \leq 0.05$), and between VTA and AMY OT levels (F (1,8) = 21.382, $p \leq 0.001$).

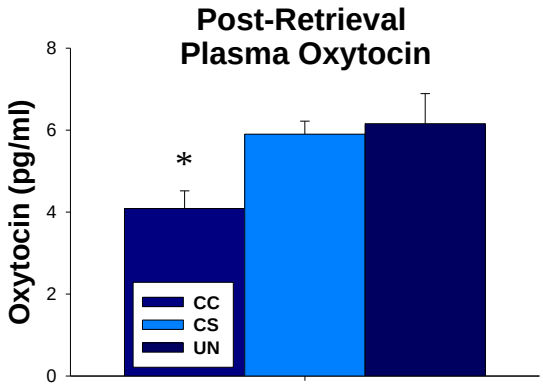

Figure 10 Post-Retrieval Test Plasma Oxytocin Levels.

Data is presented as means ± SEM. Samples were collected immediately following the retrieval test. CC-treated dams exhibit reduced levels of plasma OT compared to both UN and CS-treated dams (* = p≤ 0.05)

4.3.2 Experiment 2: Stress Response and Endocrine Measures

Open Field Test

Baseline

Analyses of cohort data indicated significant effects between cohorts in a number of behavioral variables including center duration (F (8,241) = 5.84, $p \leq 0.001$), center frequency (F (8,241) = 5.89, $p \leq 0.001$), center velocity (F (8,241) = 2.96, $p \leq 0.005$), center distance traveled (F (8,241) = 6.29, $p \leq 0.001$) and wall duration (F (8,241) = 4.08, $p \leq 0.001$). Figures can be found in the Appendix D; Figure 34.

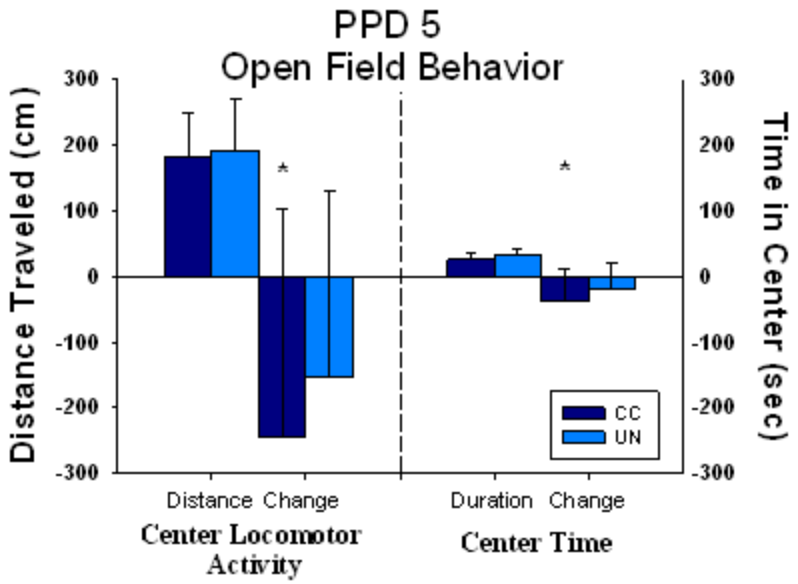

Figure 11 Open Field Behavior on PPD5.

Data is presented as means ±SD. Locomotor activity (distance traveled) is depicted on the left. Anxiety-like behavior (avoidance of the center) is depicted on the right. Each variable is presented with group behavioral data as well as the group average of the change from baseline (PPD5-Baseline). This results in negative values if there was a reduction in behavior in the postpartum. CC-treated dams differed significantly from their own baseline measures (*= $p \leq 0.005$).

PPD5

There were no treatment group differences between CC-treated and UN dams on OFT behavior or autonomic responses on PPD 5. However, when compared to baseline measures (Figure 11), Wilcoxon ranked sum tests indicate that the CC-treated group had shorter center duration ($z= -2.48$, $p\leq 0.05$), less distance traveled in the center ($z= -2.04$, $p\leq 0.05$), slower center velocity ($z= -2.23$, $p\leq 0.05$), and a trend toward less total distance traveled in the wall compartment ($z= -1.92$, $p \leq 0.06$).

CORT Response to OFT

At baseline, CC-treated dams show a trend for lower CORT levels compared to UN dams ($z=1.753$, $p\leq 0.08$). Following the OFT, Type 1 dam groups did not differ on CORT (Figure 12 A), however CC-treated dams did show a significant increase in CORT levels following the OFT ($z= 2.073$, $p \leq 0.05$). As a control for behavioral testing, Type 3 dams were tested for CORT levels at corresponding times of day but without having performed the behavioral tests. There were no differences between treatment groups or the first two time points. Type 3 CC-treated dams have significantly lower CORT levels compared to UN dams in the afternoon ($p = 0.02$, See Figure 12 B).

FST

CC-treated dams were immobile in the tank for a shorter duration (F (1,22) = 5.771, $p \leq 0.05$, see Figure 13) compared to UN dams. Experimental procedure (Type) also affected FST behavior. Type 1 dams (tail blood draws) were immobile less often (F (1,22) = 9.295, $p\leq 0.01$) and for a shorter duration (F (1, 22) = 9.672, $p \leq 0.01$) compared to Type 2 dams (no tail blood draws).

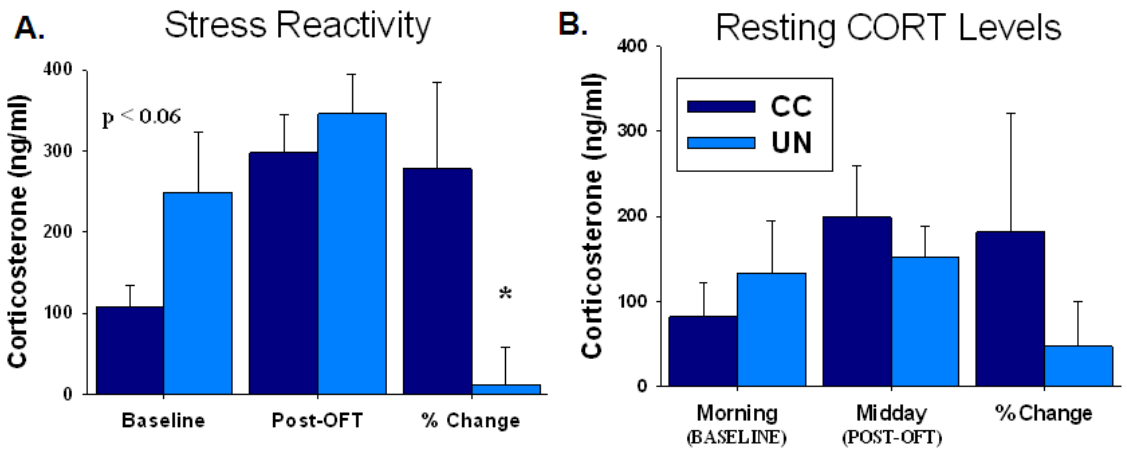

Figure 12 Corticosterone Levels at Baseline and Post-OFT.

Data presented as means ± SEM. Percent (%) change indicates group average for change between baseline and post-OFT CORT levels. A.) Stress reactivity of Type 1 dams before and after OFT. CC dams showed lower baseline $p < 0.06$. CC dams show a significant change CORT levels as a group (*=$p \leq 0.05$). B) Circadian Rhythm of Type 3 dams at same time points.

FST Oxytocin and Corticosteron Response

All hormonal data is represented in Figure 14. A two-way ANOVA (Treatment X Type) revealed no interaction but a significant main effect of Type (F (1,22) = 7.006, $p \leq 0.01$) such that FST exposure increased OT levels in all dams. There was also a main effect of treatment (F (1,22) = 5.145, $p \leq 0.05$) such that CC-treated dams had higher OT levels than UN dams. A two-way ANOVA (Treatment X Type) revealed that CORT levels were significantly raised in all dams by exposure to the FST (F (2, 22) = 26.612, $p \leq 0.001$) with no effect of treatment (see Figure 14A).

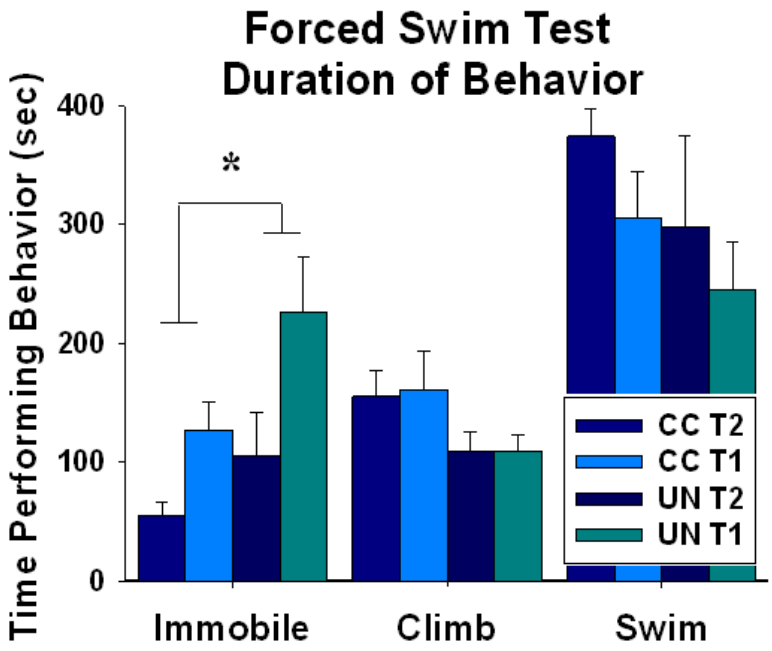

Figure 13 Forced Swim Test Behavior.

Duration of immobility, climbing and swimming behaviors for Type 1 and Type 2 UN and CC dams. Data Presented as means ± SEM. CC dams were immobile significantly less than UN dams (*= p≤ 0.05). Type 1 dams were also immobile significantly less than Type 2 dams (not indicated on graph). T: type.

Linear regression analyses indicate that OT and CORT have no direct relationship with each other at this time point in UN dams. However, CC dams show a strong positive relationship between the two hormones following the FST (R^2 = 0.76; $F\ (1,6)$ = 19.552, $p < 0.005$)). Correlations between the FST behaviors and plasma levels of OT and CORT revealed that a greater amount of total swim time (Swim-2 plus Swim-4; Swim-3 was not observed in any dams) is positively related to plasma OT levels ($F\ (1, 12)$ = 6.047, $p \leq 0.05$, see Appendix D; Figure 36).

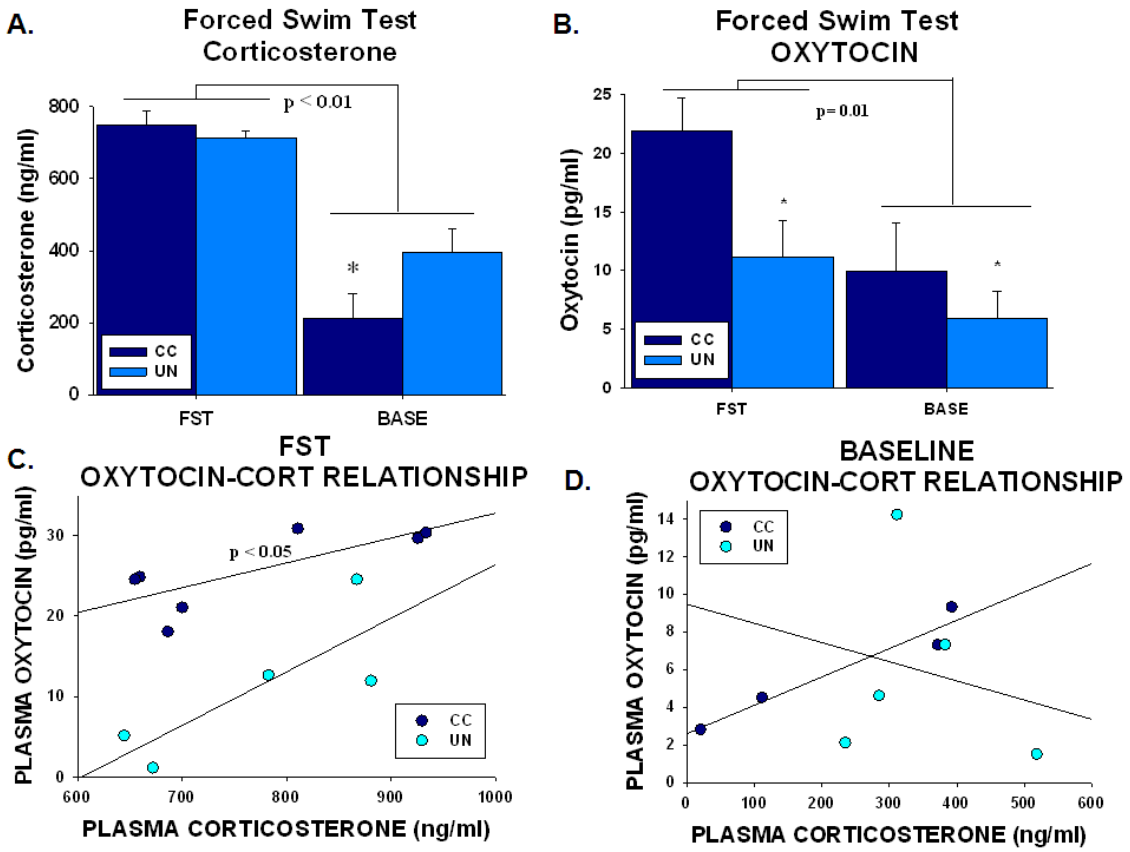

Figure 14. Endocrine Response to Forced Swim Test.

A-B.) data presented as means ± SEM. Type 1 and Type 2 pooled for analysis of FST endocrine data. A.) CORT levels increased in response to the test ($p \leq 0.001$). CC-treated dams had lower CORT at rest than UN dams (Type 3; $p \leq 0.05$). B.) OT levels increased in response to FST ($p \leq 0.001$; indicated by bar lines. CC-treated dams had higher levels compared to UN (*= $p < 0.05$), C.) Relationship between plasma OT and CORT following FST. CC-treated dams show a significant positive correlation ($p < 0.05$). D.) Relationship of OT and CORT at rest, CC-treated dams shows a significant positive relationship ($p < 0.05$), UN dams show no relationship.

Brain OT Following FST

Type 3 dams showed a trend for higher hypothalamic OT levels compared with Type 1 and Type 2 dams regardless of treatment ($F\ (2, 25)$ = 2.5775, p = 0.09). There were no differences found in AMY. No significant relationships were observed between hypothalamic and AMY OT levels. (see Appendix D; Figure 37)

4.4. DISCUSSION

We observed that CC-treated dams had lower plasma OT levels compared to UN and CS-treated dams following the retrieval test. This is consistent with observations following maternal-infant interactions in drug-abusing women (Light et al. 2004). Our data on the lack of a correlation between plasma OT and CORT levels in the postpartum also parallels what is observed in humans (Handlin et al. 2009), suggesting this is a valid translational biomarker. There was no relationship between plasma OT levels and retrieval latencies, suggesting that plasma OT levels may not be as critical to retrieval behavior as OT levels in specific brain regions. This does not rule out any role it may play in other MBs in other contexts. It must be considered that OT has a half-life of 3 minutes (Forsling et al. 1973), and that most retrieval behavior was completed within 5 minutes of the test starting. This results in an interval of at least 10 minutes before we collected samples, during which, pups may have begun suckling, thus altering OT levels. Therefore, it is possible that in rodents, plasma OT is simply a metric for the proximity to the last nursing bout, although whether pups were nursing before separation was not recorded. It is possible that pups were less likely to initiate suckling with CC-treated dams. This hypothesis is supported by the idea that CC-treated dams exhibited a greater number of behavioral transitions (see Chapter 4), which could disrupt pup attachment.

There were no significant differences between treatment groups in OT levels in any brain region, similar to previous studies that have investigated levels at this time point following maternal aggressive testing (Lubin et al. 2001), and may not be surprising given there is no change in OT mRNA by PPD 6 in CC dams (Jarrett et al. 2006). It is interesting to note that CC-treated and CS-treated dams show greater coordination of OT levels between brain regions compared to UN dams. OT levels in separate brain regions have previously been shown to be independently regulated (Johns et al. 1997), therefore the reason for this increased integration within the CS-treated and CC-treated dams remains unclear, although it may be a response to

stress during pregnancy. Our measures of peripheral and central OT collected simultaneously and lacking significant correlations suggests that plasma OT does not serve as a biomarker for central OT.

As we do not know what OT levels were like prior to testing, it cannot yet be determined whether plasma OT levels can explain these results. Future studies using microdialysis during a test could better assess the role of central OT in these behaviors. These results suggest that perhaps by PPD 5 central and peripheral levels of OT are less critical for the maintenance of MBs, which may rely more heavily on early postpartum learning, and that future studies should investigate earlier times in the postpartum period.

Nonetheless, taken together with the plasma data, these results suggests that CC treatment especially, and CS treatment to a lesser extent, may moderate site-specific OT release during a pup-interaction task. The exact mechanisms through which gestational treatment may alter regulation remains unclear, but changes in monoamine reuptake blockers have been shown to impact OT release in a similar manner (Johns et al. 2005), and thus provide an excellent starting point for future research.

Although the differences in CORT levels were not different between the dam groups, it should be noted that these levels are approximately twice that of baseline (see Figure 14A and Figure 32 in Appendix D) for all PPD 5 dams. This suggests that dams were stressed by the procedure which could explain some of the variability in behavior (see Chapter 3). Stressful environments can disrupt MBs and both peripheral and central OT levels, indicating that perhaps to the interpretation of these results may be limited to stressful parenting situations and that future studies investigating these hormonal signaling in more typical environments for MB may prove more useful to understanding differences in behavior between gestational treatment groups.

Anxiety, Depression and Stress Response

CC treatment results in subtle differences in anxiety-like and stress-coping behaviors, as well as important differences in endocrine stress signaling in the early postpartum period. Many of these differences are similar to those observed during cocaine withdrawal at other reproductive stages (D'Souza & Markou 2010). Increased plasma OT has been seen in depressed women (Cyranowski et al. 2008), and blunted HPA signaling is also a hallmark of postpartum depression (Brunton & Russell 2008). Taken together with the social deficits observed in maternal-infant interactions in dams following gestational CC treatment, another key symptom of postpartum depression, further investigation into other affective behaviors and their neurobiological correlates is warranted.

We did not see differences in the direct comparisons of OFT behavior data, primarily because the variability was very high within the groups. One source of variability is the timing of testing across the year of data collection. The baseline OFT differences observed between cohorts could be related to a variety of factors. For instance, a number of animal handlers performed the tests and the composition of these handlers changed throughout the year of testing. Although these were experienced animal handlers and rats were habituated to handling, animal handlers can affect anxiety-like behavior (Lewejohann et al. 2006). Alternatively, another explanation for time-dependent changes may be the building construction occurring nearby to the animal colony room. Animals tested during the period predicted to be the most disruptive phase of this construction (Cohort 7) actually showed greater center time and locomotor activity. Although the linear models used to analyze the postpartum OFT data controlled for cohort of baseline measures, there could be remaining environmental stimuli that are subtly different for each animal and hence increasing variability in the postpartum.

Characterizing changes in individual animal behavior proved to be highly informative in understanding how anxiety-like behaviors developed across the postpartum period, compared to

simple postpartum group comparisons. On PPD 5, CC-treated dams exhibited an increased anxiety-like profile compared to baseline, an effect not observed in UN dams. Cocaine withdrawal-induced anxiogenesis typically peaks 3-5 days following the last drug administration in non-lactating rats (D'Souza & Markou 2010), suggesting that CC-treated dams may be undergoing withdrawal from cocaine treatments that ended on their GD 20 (1-2 days prior to PPD 1).

An increase in anxiety-like behaviors is consistent with clinical literature reporting postpartum anxiety in drug-abusing women (Light et al. 2004). Although in the human literature, anxiety in the postpartum period is commonly associated with prepartum anxiety, it has been observed that anxiety can spontaneously occur postpartum (Reck et al. 2008). One possible mediator of anxiety would be changes in HPA reactivity (Holsboer & Ising 2008). CC-treated dams showed an increase in CORT in response to the OFT, a response that was absent from UN dams, suggesting that this test was more stressful to CC-treated dams. This change was not due to a typically occurring diurnal rhythm as CC-treated dams that did not undergo OFT testing did not show a similar increase in CORT. Additionally, CC-treated dams show differences in baseline plasma OT (Figure 14), which has also been tied to anxiety (Tops et al. 2007). High anxiety levels and stress reactivity can negatively impact MBs, thus potentially treating anxiety may alleviate some deficits in MB observed following CC treatment.

CC treatment alters anxiety-like behavior dynamically across the early postpartum period (see Appendix D; Figure 32), with decreased anxiety occurring early and increasing anxiety observed on PPD 5. Whether this increased anxiety continues to be observed during the stress hyporesponsive stage of the postpartum period, typically observed from PPD 7-21 (Slattery & Neumann 2008), should be investigated to determine what long-term effects may be ongoing in the postpartum period.

CC-treated dams also differed in their behavioral response to a highly stressful environment, the FST. CC-treated dams exhibit greater struggling or ‘proactive’ coping with the

stressful situation (Coppens et al. 2010, Pollak et al. 2010). Traditional interpretation of immobility is described as 'despair' which is decreased upon antidepressant administration (Pollak et al. 2010), does not seem appropriate given the high amount of immobility in postpartum dams compared to virgin female rats observed here and in other studies (Craft et al. 2010). There are a number of physiological reasons why immobility or a 'reactive' coping style would be an advantageous strategy for postpartum females including changes in fat content, body density, and differences in metabolic and stress mobilization (Augustine et al. 2008). This suggests, nonetheless, that the neurobiology underlying FST behavior has been altered by CC treatment.

It has been previously shown that in male rats an initial exposure to uncontrollable swimming results in massive release of OT into the AMY, SON (measured by microdialysis) and plasma (Wotjak et al. 2001, Engelmann et al. 2006, Ebner et al. 2005). We observed that in all dams an increase plasma OT, however compared to baseline control animals they showed lower hypothalamic levels following the FST, suggesting that hypothalamic release into the plasma. Following the FST, CC-treatment dams showed much higher OT levels compared to UN dams which could be a greater stress response since OT is known to be released into the blood stream in response to stress in and can temper circulating CORT levels (Ditzen et al. 2009, Neumann et al. 2000). SON OT levels appear to correspond with the degree of uncontrollability of the behavioral context (Engelmann et al. 2006), indicating that future studies using more anatomically specific measurement techniques may reveal changes it central OT that explain the greater behavioral stress reactivity in CC-treatment dams compared to UN dams.

In addition to the increased OT following FST, we observed that CC-treatment dams show a significant relationship between plasma OT and plasma CORT levels that UN dams do not. Although this is based on a small sample size, it indicates that in response to stressful stimuli, the regulation of the hypothalamus, specifically the parvocellular cells of the PVN, may be altered in CC-treatment dams. Future studies may focus more attention on these cells to better understand

changes that may occur following CC treatment.

Taken together, these data indicate that CC treatment during pregnancy alters peripheral endocrine signaling in a behaviorally context-specific fashion. Such disruptions likely interact and modulate the dams' physiological state and thus her behavior. The finding that the changes are dependent on the environment, suggest a complex tuning of these endocrine systems, not just a simple knock-down of their function. Mechanisms for this regulation are discussed in detail in Chapter 7.

CHAPTER 5. EFFECTS OF GESTATIONAL COCAINE ON OLFACTORY PREFERENCE FOR PUP CUES AND NEURONAL ACTIVATION

5.1. INTRODUCTION

Olfaction is the primary sensory system for rodents to perceive the environment. Rat dams are exposed to a number of novel olfactory cues originating from pups and placental fluids during and immediately following parturition. The neuroanatomical circuitry central to olfaction includes the main olfactory bulb (MOB), vomeronasal organ or accessory olfactory bulb (AOB), olfactory tubercle and piriform cortex. This circuitry's function is critical for social interactions, including MB (Levy et al. 2004). Rat dams prefer odors associated with pups or other rat dams, and disruption of the olfactory system can affect maternal retrieval (Bauer 1993, Bauer 1983, Magnusson & Fleming 1995, Kinsley et al. 1995). The olfactory sensory circuit activates in response to pups and pup cues as measured by c-Fos and functional magnetic resonance imaging (fMRI) (Fleming & Walsh 1994, Ferris et al. 2005). Lesioning of the AOB disrupts licking behavior (Brouette-Lahlou et al. 1999), and lesioning the MOB increases the latency to retrieve pups (Kolunie et al. 1994, Fleming & Rosenblatt 1974, Fleming & Rosenblatt 1974, Benuck & Rowe 1975), suggesting an important role for olfaction in the appetitive aspects of MB.

CC-treated rat dams exhibit reduced licking compared to control dams (Johns et al. 1997), and CC-exposed pups elicit less maternal care than control pups, suggesting that PC exposure may impact olfactory cues that induce licking. However, very little is known about how prenatal or gestational exposure to cocaine may affect pup-produced olfactory cues or dams' response to them.

Disruption of olfactory bulb function may have many downstream effects of behavior given

its direct neuronal connections. The AOB sends glutamatergic projections to the medial amygdala (MeA), cortical amygdala (CoA), and bed nucleus of the stria terminalis (BNST), while the MOB projects to the CoA, piriform and entorhinal cortices as well as the SON (Yoon et al. 2005, Yang et al. 1995). These direct connections suggest that olfactory cues can directly affect fear and social behaviors. Numan and colleagues have postulated that this circuit controls pup avoidance in virgin rats and approach in postpartum rats (Numan 2007). The proposed circuit describes MeA neurons sending excitatory projections to anterior hypothalamic nuclei (AHN) and ventromedial hypothalamus (VMH). AHN neurons project to the periaquaductal gray (PAG), which plays a role in avoidance response to pup stimuli in virgin rats (Numan 2007). However, following parturition, both the medial preoptic area (MPOA) and the BNST can act to inhibit AHN and PAG. Simultaneously, the MPOA and BNST can instead activate the VTA, which releases DA into the NAc and drives motor responses toward pup approach (Numan & Stolzenberg 2009). This indicates that understanding how the NAc responds may elucidate an underlying mechanism for differences in MB toward specific to pup cues.

OT is important for coordinating the maternal response through a number of these brain regions (Lee et al. 2009). OT enhances the development of olfactory-based social memory (Engelmann et al. 1998, Crawley et al. 2007), with increased signaling in the MOB and MeA having been shown to be especially important (Ferguson et al. 2001, Larrazolo-Lopez et al. 2008). OT has recently been shown to be critical for diminished fear responses to predator odors in lactating dams (Febo et al. 2009). CC treatment during gestation has been shown to decrease OT in a number of brain regions (Johns et al. 1997), and thus could be interfering with olfactory perception or early olfactory learning in the postpartum, although this has yet to be directly tested.

Given the importance of olfactory perception to MB, and the deficits observed in maternal-infant interactions these studies aimed to a. determine if CC-treated dams compared to CS-treated and UN dams differentially prefer pup-produced olfactory stimuli (urine) from CC-exposed

versus UN prenatal exposure conditions. b. To determine if CC-treated dams exhibit differential neuronal activation (c-Fos) patterns in response to pup olfactory stimuli from CC-exposed and UN pups.

CC-treated, CS-treated, and UN rat dams (treated as in Chapter 3) were tested for behavioral preference for a pup urine stimulus from unfamiliar CC-exposed or UN pups on an olfactory choice test on PPDs 1 and 3. Preference for their own biological pup urine versus another treatment pup was examined on PPD 5. Separate groups of CC-treated and UN dams were tested for c-Fos expression in brain regions important for maternal response following exposure to either CC-exposed or UN pup urine on PPD 5.

I hypothesized that all dams would show a preference for UN exposed pup urine on PPDs 1 and 3. I also hypothesized that on PPD 5, UN and CS-treated dams would prefer their own pups' urine, but CC dams would not. CC dams were expected to show lower c-Fos expression in response to pup urine in maternal motivation circuitry compared to UN dams.

5.2. EXPERIMENTAL DESIGN AND METHODS

These dams were same as those used for Retrieval Preference testing. Breeding and gestational treatment methods can be found in Chapter 2.

5.2.1 Experiment 1: Olfactory Preference

Olfactory Preference Testing (OPT) Chamber

The testing chamber (See Figure 15) consisted of a start box (20 X 22 cm) with a retractable door (seen in red) that separated the start box from the Center (immediately outside of Start box and between the two alleys (13 X 21 cm). Two alleys (65 X 11 cm) separated by a wall (seen in blue), are divided into Distal or Proximal compartments. Distal portions of each alley were defined as the half of the alley furthest from the olfactory stimulus; Proximal portions of each alley were the half of the alley closest to the olfactory stimulus. All walls were 31 cm high and covered in opaque paper to prevent side preferences from occurring and encourage exploration. A

tea strainer containing a cotton ball with either pup provider or experimental dam pup urine olfactory stimulus hung at the end of each alley 15 cm above the floor. A mirror was affixed above the chamber to allow videotaping without creating a shadow within the chamber.

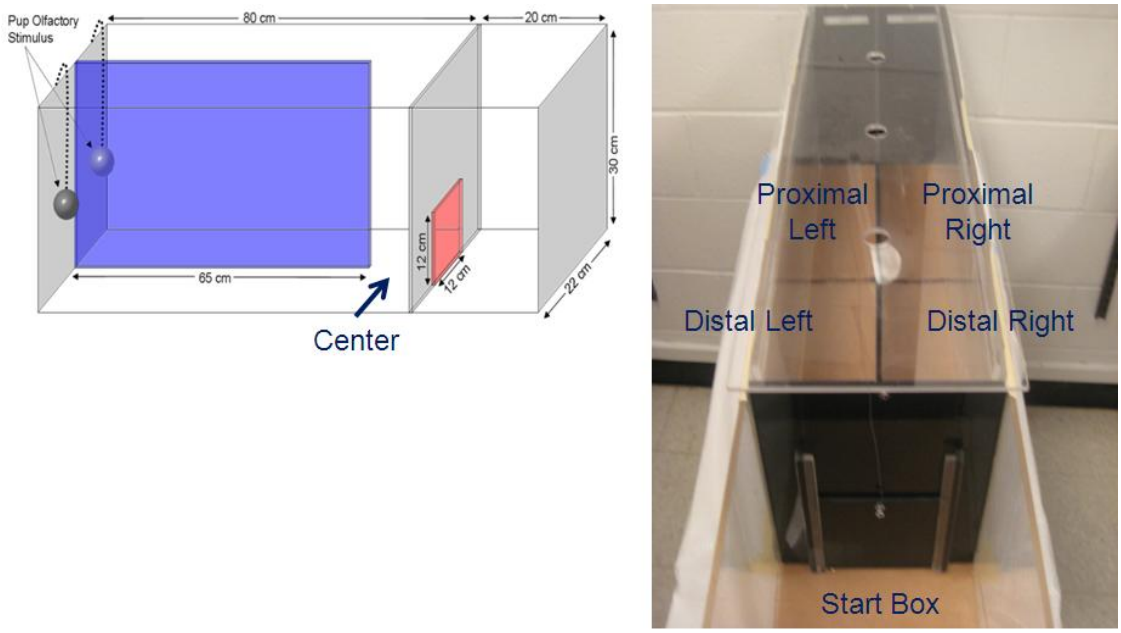

Figure 15 Olfactory Preference Test (OPT) Chamber.

On left, diagram of chamber, depicting measurements, placement of olfactory stimulus containers, and the Center compartment. On right, photograph of chamber, depicting other compartments (see text for details).

Olfactory Response Testing

On GD 18, all dams were brought to the test room and placed in a novel chamber (retrieval test apparatus, see Figure 6) for 10 minutes and allowed to search for fruit flavored cereal as a test for normal olfactory response. Pilot studies indicated that pregnant dams will spontaneously consume this cereal when presented in the home cage or novel cages. There were no deficits in olfactory ability as measured by this task due to gestational treatment conditions (see Table 5 in Appendix C).

Habituation

On GDs 18 and 19, all dams were habituated to the olfactory test chamber, using cotton

balls without urine placed in the tea strainers. During habituation, the dam was first placed in a fresh cage which had never housed pups in the test room for 5-15 minutes. On GD 18, the dam was placed in the OPT with the start box door open and allowed to investigate the chamber freely for 10 minutes. Pilot data indicated that a majority of dams finished exploring and were resting by the end of the timed habituation session. On GD 19, the dam was placed in the closed start box for one minute before the door was opened and she was allowed 10 minutes to investigate the apparatus while the session was videotaped to determine baseline left/right side preferences (see Chapter 2 methods). Urination and defecation by the dams were also recorded to assess autonomic activity in the OPT chamber.

Olfactory Testing Procedure

On PPDs 1, 3, and 5, the experimental dams and pup-provider dams were brought to the pretest preparation room (Chapter 3, see Figure 6). Dams were separated from pups for approximately twenty minutes, while urine was collected from pup-provider pups. On PPDs 1 and 3, urine was collected (see methods, Chapter 6, pup urine collection) from four UN and CC-treated pup-provider dams' pups for the olfactory stimulus. On PPD 5, the cotton balls contained urine from the test dam's pups or urine from a pup-provider pup of the opposite treatment (i.e. UN urine versus CC-treated, own versus other-Figure 16). Twenty µL (10 µL Male/10 µL Female) of urine from the different treatment or control pups was pipetted onto each cotton ball. Five µl of urine has been shown increase neuronal activation and elicit behavioral responses in rodents (Honda et al. 2008), and was the amount we could consistently collect from pups of all ages. The cotton balls were placed in the two tea strainers in the alleys of the testing chamber. The side in which the CC-exposed urine cotton ball was placed counterbalanced across test days and test dams. The dam was placed into the start box for one minute. At that point, the door to the start box was opened and the dam was allowed to enter the test chamber for 10 minutes. The session was videotaped for later analysis. Urination and defecation by the dam were recorded in

each compartment to assess autonomic activity in the OPT chamber. Following the test, the dam was returned to a resting cage (see Figure 16 below for overall experimental design). Similar to Retrieval Preference Testing, dams were exposed to pup urine from pup providers (UN/CC-exposed) on PPDs 1 and 3. On PPD 5, dams were exposed to pup urine from their own litter or litters from an opposite treatment groups.

Surrogate Pups	Postpartum day					
Experimental Dam Pups	1		3		5	
Dam treatment	Maternal Olfactory Preference		Maternal Olfactory Preference		Maternal Olfactory Preference	
CC (n=9)	CC pups	UN pups	CC pups	UN pups	CC pups	UN pups
CS (n=9)	CC pups	UN pups	CC pups	UN pups	CC pups	CS pups
UN (n=10)	CC pups	UN pups	CC pups	UN pups	CC pups	UN pups

Figure 16. Experimental Design for Olfactory Preference Testing

On PPDs 1 and 3, experimental dams (red boxes) chose between surrogate pups' urine (blue boxes) from pup-provider dams. On PPD 5, experimental dams chose between their own biological pups' urine (red boxes) and surrogate pups' urine.

Data Analysis

Timing of experimental procedures was considered important for reducing variability, thus all timing for test procedures (i.e. start time, pup separation time) were noted. There were no differences in experimental procedural times across groups. Tapes were coded as previously described (see Chapter 2-Methods). Dams were recorded as being in one of five compartments (see Figure 15); Center; Distal LEFT Alley; Distal RIGHT Alley, Proximal LEFT alley, Proximal RIGHT alley. Distances (distal/proximal) refer to proximity to the olfactory cue containers. Dams were said to have entered a compartment of the chamber if the head and both forelimbs had crossed the marked entry point. Sniffing was defined as the dams' snout in contact or close proximity to the olfactory stimulus with head motions indicative of sniffing behavior. Touching was defined as forepaw contact with the olfactory stimulus container in the absence of sniffing. To determine "Preference" for an alley, the duration of all behaviors in that alley (Distal, Proximal, Sniff, Touch) were summed. If a dam spent greater than 40% of test time in one alley

(LEFT, RIGHT, CENTER) she was defined as having preferred that side. Data was later matched to pup treatment to determine UN and CC-exposed pup alley preference.

Statistical Analysis

Baseline OPT Preference

Linear models were used to compare the frequency, duration and latency of behaviors between treatment groups. Logistical regression analyses (adjusted for alley duration) were used to test for associations between autonomic response and alley preference. Cochran-Mantel-Haenszel tests were used to compare the proportion of time dams in each group exhibited preference behavior to control for the multiple tests of independence.

Postpartum Preference

Since greater than 90% of dams exhibited a strong side preference during habituation on GD 19, linear models incorporated this initial side preference. Data from each alley side (i.e. left or right) were pooled by pup urine condition (UN, CC-treated, or CS-treated) for analysis. Due to the non-normality of the data, non-parametric tests (PPD 1 and 3; Kruskal-Wallis, PPD 5: Wilcoxon rank sum) were used to analyze differences in dam treatment condition on frequency, duration and latency measures. Wilcoxon rank sum tests were used to compare CC-exposed and UN urine alley preference (regardless of dam treatment). Fischer's exact tests were used to assess differences in the proportion of dams exhibiting any preference behavior and Chi square tests assessed the proportion of dams exhibiting avoidance behavior (no choice region of box).

5.2.2 Experiment 2: Neuronal Activation In Response to Pup Cues

Apparatus

A rectangular plexiglass cage (27cm X 50 cm) with fresh corncob bedding on the floor was used to expose dams to the pup urine olfactory stimulus (FOS test cage). Pup urine stimulus containers (plastic centrifuge tubes) were placed in all 4 corners of the cage. Each stimulus

container housed a cotton ball that had 20 uL of urine from PND 5 CC or UN pups on it.

Experimental Procedure

To habituate the dams to the chamber on GD 18 and 19 all dams were brought to testing room and placed in the chamber. On GD 18, dams were tested for olfactory ability (see above). All dams consumed cereal. Since the pups of these dams were used for other experiments (see Chapters 3, 6), on PPDs 1 and 3, dams were brought to room for approximately 30 minutes and 4 pups from each dam were removed while the dam and remaining 4 pups stayed in the home cage. No dam ever had all pups removed from the cage simultaneously until PPD 5.

On PPD 5, dams were transported to the test room, all her pups were removed and she was placed in the FOS testing apparatus. The dam in the Fos test cage was placed in a separate test room with no other animals present for 6-8 hours (long enough for c-Fos expression due to pup separation to have diminished (Kovacs 2008). Four pup-stimulus containers (one in each corner) were then added to the cage. The olfactory stimulus containers had either CC-exposed or UN pup urine. The experimenter held the stimulus containers to the dams' snout for 30-45 seconds to make certain they sniffed the stimuli, and dams did not avoid the stimulus or show signs of fear (i.e. freezing). Dams were left in the chamber with the olfactory stimulus containers for 120 minutes, removed and immediately transcardially perfused (see Chapter 2-Methods). All dams were noted as continuing to investigate the olfactory stimulus containers for at least 1 minute after exposure; however, total length of investigation time was not recorded afterwards.

Tissue Treatment and Analysis

Following perfusion, tissue was prepared and immunohistochemistry staining for c-Fos protein was performed and unbiased stereological counting procedures were used to estimate the total number of stained nuclei as described in Chapter 2-Methods. A two-way ANOVA (dam treatment x pup treatment) was employed to compare the number if c-Fos stained nuclei.

5.3. RESULTS

5.3.1 Experiment 1: Olfactory Preference

OPT Habituation

Dam treatment had no measurable effect on the frequency, duration or latency of any baseline behaviors in the OPT chamber. Dams spent the majority of the time either in the Center (See Figure 15, left) or in the Proximal compartments of alleys (see, Figure 15, Right; Appendix E; Figure 39 for data). The total frequency of behavior across the ten-minute test was interpreted as a measure of locomotor activity. There was no effect of dam treatment on this measure, suggesting that there was no effect of dam treatment on ability to explore the OPT chamber.

Preference was operationally defined as spending greater than 20% more time in an alley compared to either the other alley or the center (i.e. 40% or higher). The majority of dams exhibited a preference for one compartment (see Appendix E; Figure 38), however, no dam group showed a definitive side preference for one alley of the OPT chamber over the other, indicating that the chamber itself is unbiased, and thus was an effective apparatus for this test. Dams did not differ in their distribution of side preference (Cochran-Mantel-Haenszel test, $p= 0.45$; see Appendix E; Figure 38).

There were no treatment related differences on urination or defecation in the OPT chamber and no relationship between alley and autonomic response. Taken together these results give us confidence that OPT chamber does not induce undue fear responses and its design does not bias behavior toward one side of the other.

Postpartum Testing

There were no differences in the amount of time spent investigating (sniffing plus touching) the olfactory stimulus container between groups or across days. Graphs for specific behaviors can

be found in the Appendix. E; Figure 40. A treatment X day interaction (F (4,1124) = 2.705, $p \leq 0.05$), was followed by post hoc hypothesis tests indicating that on PPD 1, CS-treated dams were less active than CC-treated dams (F (1,1132) = 6.335, $p \leq 0.05$). On PPD 3, UN dams were less active than CS-treated dams (F (1,1124) = 8.920, $p \leq 0.005$) and CC-treated dams (F (1,1124) = 13.736, $p \leq 0.01$). On PPD 5, UN dams were less active than CC-treated dams (F (1, 1124) = 9.658, $p \leq 0.005$; Appendix E, Figure 42). There were no significant differences in autonomic response across test days. Since there was no association with side preference and autonomic measures during habituation, these analyses were not performed for postpartum testing.

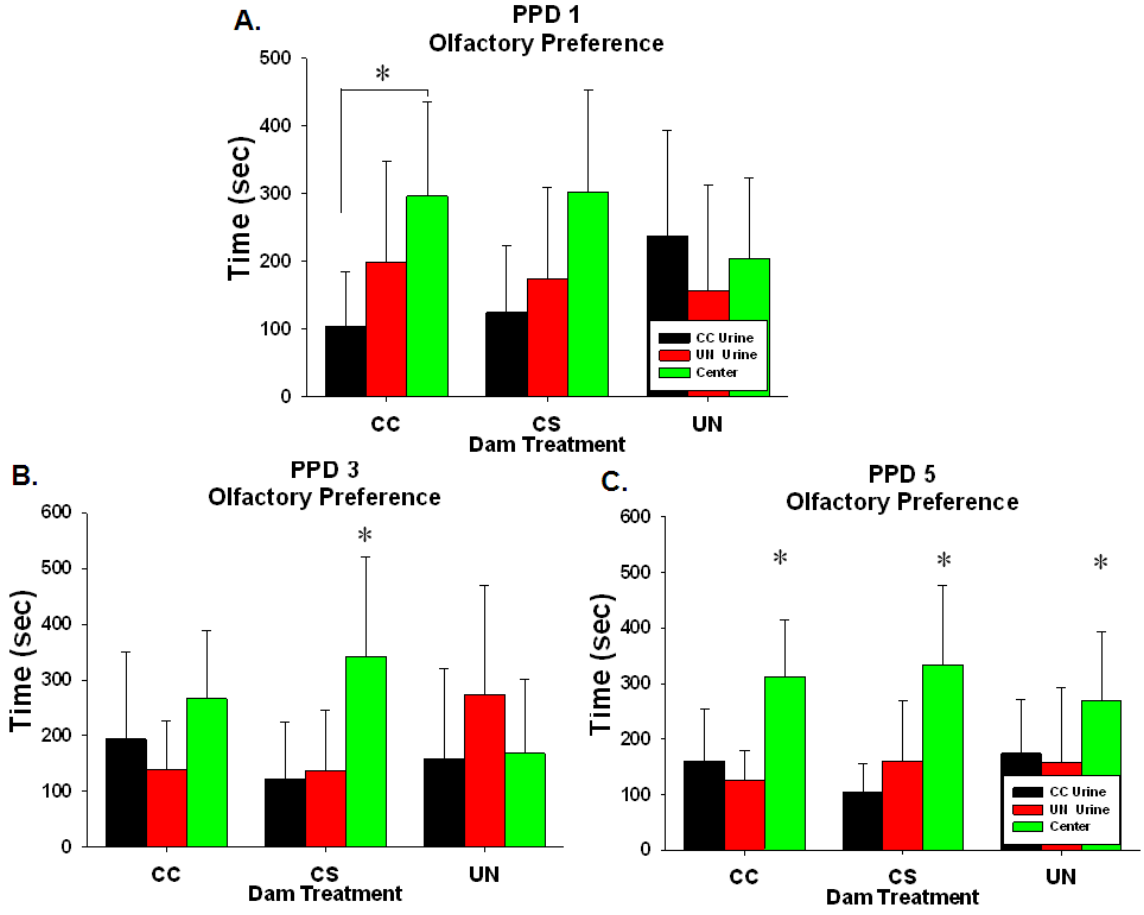

Figure 17. Olfactory Preference for Urine Cues.

Time spent in each alley presented as treatment group means ± SEM. *= Within Treatment group difference between compartments $p \leq 0.05$. CC-treated dams spent more time in center compared to CC-exposed pup alley on PPD 1 ($p < 0.05$). CS-treated dams spent more time in Center than either pup alley on PPD 3 ($p < 0.05$). All dams spent more time in Center on PPD 5 compared to pup alleys ($p < 0.05$).

Pup Urine Effects

PPD1

UN dams spent a greater duration in the distal CC-exposed alley compared to both CS-treated ($p < 0.05$) and CC-treated dams ($p < 0.02$). CC-treated dams 'touched' the CC-exposed stimulus over a shorter duration compared to CS-treated dams ($p < 0.04$), but touched more frequently compared to both CS-treated ($p < 0.02$) and UN dams ($p < 0.03$). Preference scores for CC-exposed, UN and Center compartments indicated that CC-treated dams preferred the center compartment more than the CC-exposed alley ($p < 0.01$, See Figure 17). CS-treated and UN dams had no preference for either alley or the center compartment.

PPD3

CC-treated dams sniffed the CC-exposed urine container more often ($p < 0.03$), and showed a corresponding increase in the frequency of being in the proximal CC-exposed alley ($p < 0.03$). CS-treated dams preferred the center to both the CC-exposed pup alley ($p < 0.002$) and the UN alley ($p < 0.02$) and CC-treated dams showed a trend to prefer the Center compared to CC-exposed alley ($p < 0.07$). UN dams showed no clear preference for either alley or the center.

PPD5

There were no significant differences between dam treatment groups on any individual behavior on PPD 5. However, all dams preferred the center compartment to the UN alley ($p \leq 0.05$) and the CC-exposed alley ($p \leq 0.05$).

5.3.2 Experiment 2: Neuronal Activation

Survey

Exposure to pup urine resulted in increased c-Fos activation in a number of brain regions known to be involved in olfactory processing, maternal behavior and motivation. We observed

staining in olfactory tubercle, piriform cortex, mPFC, infralimbic cortex, MPOA, BNST, lateral habenula, HIPP, AMY, periaquaductal gray and VTA. Staining was highly variable across groups. However, given the importance of the NAc to the association of cues and motivated behavior, it was chosen for initial quantification.

Activation

When estimated total c-Fos positive cells in the entire nucleus accumbens were measured using unbiased stereology (average Gundersen Coefficient of Error for both groups was 0.10), no differences were observed in the estimated population using mean counting thickness between dam treatment or pup prenatal exposure condition (see Table 1).

	Pup Treatment					
Dam Treatment	UN			CC		
UN	22191.52	±	4291.261	40223.39	±	8501.622
CC	34973.82	±	10734.75	37733.65	±	15762.09

Table 1. Total Estimated Cell Count Following Pup Urine Exposure.

Data presented as means ± SEM for each group. Data calculated as population estimates using mean section thickness with counts.

5.4. DISCUSSION

On PPD 1 CC-treated dams avoid CC-exposed pup urine at the time when UN dams are showing the greatest interest in the same stimulus. CC-treated dams, and to a lesser extent CS-treated dams, may find the chemical cues in the PND 1 urine more aversive, as it is unlikely an olfactory ability change (see Appendix C; Table 5). Dopamine presence in the MOB is important for the initiation of maternal behavior on PND 1 (Keverne et al. 1993), but CC treatment does not change levels of DA or its metabolites in the MOB. However, CC treatment does result in decreased production of new MOB neurons and decreased olfactory receptor variance, as well as increased expression of metabolic, stress response, and neuroplasticity genes (Xu et al. 2010). These effects of cocaine may inhibit the already substantial changes that occur in the olfactory

bulb around parturition in the rodent (personal communication, Treloar H.). OT can affect both pre and post-synaptic function in olfactory bulb neurons (Osako et al. 2001), contributes to accessory bulb dependent social learning (Fang et al. 2008), and is decreased in several brain regions in the early postpartum period by CC treatment (Johns et al. 1997).

The constituents of the urine are different in CC-exposed pups (see Chapter 6) which could directly impact this behavior in dams. Specific attributes of the urine which may have affected preference are fully described and may have been a partial mechanism for behavioral differences in dam preference on this task. Future studies may investigate olfactory bulb neurochemistry on PPD 1 following CC treatment to more fully understand these results.

CC-treated dams show a greater frequency of behavioral switching more often than CS-treated or UN dams (see Appendix E, Figure 42), which potentially indicates an important difference in decision-making skills. This is a common symptom of drug abuse and withdrawal (George & Koob 2010), and may be important for exhibiting a preference response as measured in these tests. Analysis of the habituation phase of the test suggests that CC treatment did not alter how pregnant dams responded to novel environments during late gestation. This may be due to general lack of stress response observed in late pregnant rats, and very low levels of locomotor activity.

Importantly, dams showed no differences in investigating the olfactory stimuli containers. This suggests that preference behavior was based on their response to olfactory cues. It was believed that the olfactory stimulus was strong enough to induce behavioral response since it is four times greater volume than the amount used to produce behavioral response and neuronal activation in mice (Honda et al. 2008). However, dams did not spend a large proportion of time (less than 10%) investigating the stimulus containers. Instead, dams spent a greater amount of time in the center compartment compared to all other compartments. Although this may be due to the necessity of passing through the center to reach the opposite alley, it also suggests that a stronger stimulus may have elicited a greater behavioral response.

The decrease of a preference choice across days in UN dams suggests that a urine olfactory cue may be most salient at the initiation of maternal care and as time progresses other cues take on more or a different value to the dam. Future studies could employ modified CPP testing to see if this olfactory cue may elicit reward seeking behavior. CC-treated and CS-treated dams spent the majority of their time in the center compartment regardless of the test day, suggesting pup urine cues may not be motivating to these dams at any time point, and that they may need a combination of cues to develop preference for pup stimuli.

Neuronal Activation

We did not observed the expected decreases in c-Fos activation in the NAc of CC-treated dams which might argue against a rewarding value of specific types of pup urine, but this may be explained by a general lack of salience of this cue at on PPD 5. If our data had been collected on PPD 1, when there were differences in olfactory preference we may have seen significant effects in the NAc. Although cocaine treatment is known to reduce the incentive salience of natural rewards such as sucrose (D'Souza & Markou 2010), and the NAc contains cells that respond specifically to cocaine versus sucrose reward (Carelli & Wondolowski 2003), it is possible that pup urine is such a strong cue to both UN and CC-treated dams that no decreased activation was observed. Alternatively, pup urine may not be a strong enough cue to elicit strong and sustained neuronal activation. Interpretation of c-Fos results is limited by the immense variability observed in staining. Variability may be influenced by the 'amount' of stimulus each dam experienced. Although, experimentally the dams were made to smell the olfactory cue container for 1 minute, there is no record of how much time they spent actually sniffing the container. Pilot studies suggest that there are no effects of dam or pup treatment on c-Fos activation in the VTA (see Appendix E; Table 9), although if the NAc is separated into core and shell we may begin to find differences (see Appendix E; Figure 43). Retrieval behavior testing suggests there are not major differences in overall appetitive behavior toward pups on PPD 5. My hypothesis was based on the

idea that CC-treated dams would exhibit less appetitive behaviors towards pup cues, and the NAc is especially important for appetitive behavior, but retrieval behavior testing (see Chapter 3) suggests there are not major differences in overall appetitive behavior toward pups on this day. Perhaps investigation of more 'maternal behavior' specific regions such as the MPOA or BNST may show differences.

CHAPTER 6. EFFECTS OF PRENATAL COCAINE ON PUP-PRODUCED CUES AS MESCHANISMS OF MATERNAL RESPONSE

6.1. INTRODUCTION

Two of the most significant pup cues for maternal care are auditory and olfactory cues. Pup-produced olfactory cues are mainly attributed to excretions, either from urine, feces or the preputial gland. These excretions are released by maternal licking in the early neonatal period and rat dams consume pup urine for their own water and electrolyte balance (Capek & Jelinik 1956, Friedman et al. 1981, Gubernick & Alberts 1983). Urine is a potent social signal in rodents, involved in interactions as diverse as territorial aggression, sexual initiation, and maternal behavior (Tirindelli et al. 2009). There are known to be several pheromones that can directly influence behavior in adult urine (Cotton 2007), however whether these exist in infant pup urine remains to be seen. Urine is not the only odiferous compound dams are exposed to from pups. Pups commonly excrete feces simultaneously with urine, which may mask many urine odors. Additionally, rodent pups excrete a number of compounds from their preputial glands, one of which (dodecyl proprionate), is known to play an important role in maternal licking behavior (Brouette-lahlou et al. 1991). Combined these odors drive an olfactory preference in postpartum females (Bauer 1983, Bauer 1993), and may play a role in the differential licking received by males over females (Moore & Morelli 1979, Moore 1981, Moore 1985). However, very little is known about how PC exposure may affect pup-produced olfactory cues.

Ultrasonic vocalizations (USVs) are another potential pup-produced cue that could be altered by PC exposure. USVs have significant communicative value in rodents and especially to dams (Ehret 2005, Noirot 1972, Brunelli et al. 1994). Sustained high-rate USVs emitted by pups

are the most effective cry pattern for eliciting retrieval from dams (Brunelli et al. 1994, Farrell & Alberts 2002, Farrell & Alberts 2002, Zimmerberg et al. 2003). Furthermore, USVs can enhance maternal anogential licking behavior (Brouette-Lahlou et al. 1992), indicating their important interactive role in eliciting other MBs following retrieval. Crouching may also be impacted as USVs may directly stimulate prolactin secretions in dams (Stern et al. 1984, Hashimoto et al. 2001, Terkel et al. 1979). These data implicate a possible direct effect of USVs on dam behavior; however, whether CC treatment affects the dam's ability to respond appropriately to these auditory cues is currently unknown.

Given the importance of USVs and urine olfactory cues in the elicitation of maternal care, these mechanistic studies of pup cues were done as part of specific aims I (c) and III (b) to discern if either were underlying contributors to any observed deficits in MB or preference for pup stimuli. These studies aimed to determine if CC exposure could alter pup-produced cues that are relevant to maternal care by examining a.) birth weight, b.) USVs, and c.) olfactory cues. Rat pup litters (CC-exposed or UN) had USV calls recorded on PNDs 1, 3 and 5 before maternal retrieval testing (see Chapter 4). Calls were analyzed for USV characteristics to determine if specific differences are present, and correlated with maternal behavior in dams. Rat pup litters had urine collected on PNDs 1, 3 and 5 before maternal olfactory preference testing (see Chapter 6.) Urinalysis was performed on all pup urine samples to measure markers of malnutrition and physiological health of the pups.

It was hypothesized that CC-exposed pups would be smaller at birth, have altered urine olfactory cues and litter preference would be related to the smaller number and shorter duration of pup group USVs compared to control pups. Differences were expected to be greatest on PND 1 and decline across the first neonatal week. Overall gestational size and health of pups were also assessed and are reported in results.

6.2. EXPERIMENTAL DESIGN AND METHODS

6.2.1 Experiment 1: Gestational Data and Body Weights

Immediately following parturition (PND 1), pups were removed from the dams, and gestational length, litter weight, number of pups per litter, and sex ratio were recorded. One-way ANOVAs were used to assess differences between test dams (UN/CS-treated/CC-treated) and Students t tests were used to assess differences between pup-provider dams (UN/CC-treated) in gestational length, weight gain, litter weight, culled litter weight, sex ratio, individual pup weight. Chi square analysis was used to determine differences in proportions.

Experiment 2: USV Testing

USV Testing Procedure (Specific Aim I)

Pup-provider dams and their litters were brought to the preparation room. Pups were labeled with a marker by sex and treatment group and returned to their dam. Following a 15-minute reunion, pups were removed, and "nest temperature" was recorded. Pups were placed together on a warm heating pad for 20 minutes. Pups were removed from the heating pad, 'heat-pad temperature' recorded, and placed separately on a plastic sheet to allow cooling to occur for 10-15 minutes. There were no differences between the treatment groups or ages on the amount of time separated from their dam or the amount of time they were exposed to the cold (See Appendix F; Table 10).

Immediately prior to the test, 'test temperature' was recorded. Pups were placed as horizontally as possible into the Plexiglas pup containers and transferred to the testing retrieval testing chamber (see Apparatus-Chapter 3; Figure 5). Recording began immediately and lasted for 1 minute. New recordings began when dams were added to the chamber and lasted until all of the pups were retrieved. Following the retrieval test, temperature was recorded and pups returned to their home cages.

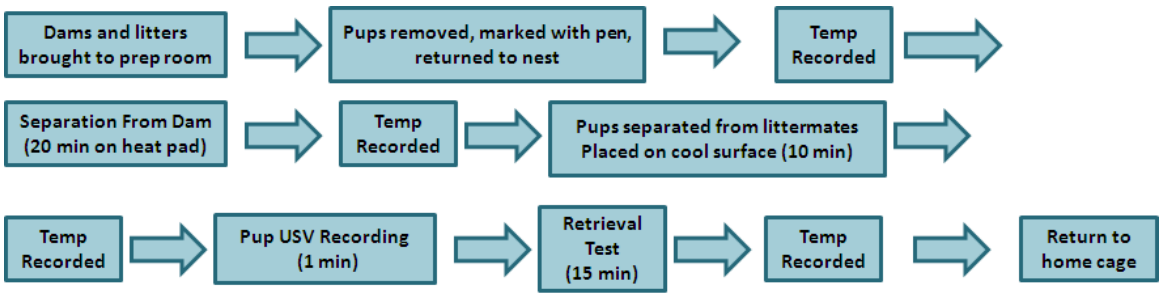

Figure 18 Experimental Procedure for Collecting Pup Temperature and USVs.

The entire testing protocol lasted approximately 1.5 hours. All pups underwent the same testing procedure, with all times of each step recorded. There were no differences in time between treatment groups or ages. The procedures resulted in pups vocalizing by the time of recording. Temp: temperature

Temperature Recording

For all temperature measurements, a single pup was randomly chosen from each litter at each time point. The skin temperature on the rear flanks was measured with a laser thermometer (Fischer Scientific). Temperature changes were calculated to determine heat loss and gain over procedural steps.

Vocalization recording

Ultrasonic recording equipment included Med Associates model ANL-932-1 ultrasound detectors sampling at a rate of approximately 30 samples per second that were connected to transducers, and then to a laptop computer. Med Associates USV software began acquisition of USVs at the session start and terminated as described above. Baseline recordings were collected on every test day to ensure there were no changes in background level of noise.

Data Analysis

Our design does not allow for the determination between individual USVs from individual pups, however, these recordings measure characteristics of the 'call' sounds the dam hears from these pups. In order to characterize these 'call' sounds, several steps were taken to clean and analyze the recordings. Ultrasonic recorded data were transferred to Excel sheets via the software

provided by Med Associates. Analyses were performed on the total ultrasonic sounds measured in the one minute session. Analyses included number, duration, and dominant frequency (the loudest frequency recorded) of any ultrasonic sound recorded regardless of whether its origin seemed to be from pups. These analyses could not differentiate background noise from actual USVs emitted from the litter of pups. Therefore, an analysis program was developed (kindly designed by A.V. Avram) using MATLAB R2010a software (The MathWorks, Inc., Natick, MA) that filtered the data to remove ultrasonic sounds that were not USVs. The operationally defined classifications are detailed below.

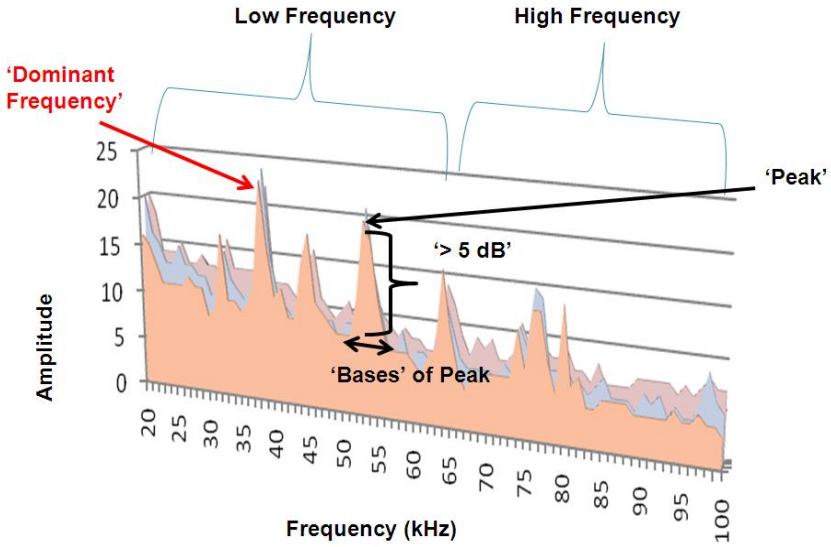

Figure 19 Example USV spectrogram used for analysis.

This graph shows the frequency (kHz) on the x –axis. Amplitude (dB) on the y-axis. A 'peak' (black arrow) in an USV is defined as a point at least 5 dB greater than the average of its 'bases'. For example this peak has a frequency of 53 kHZ and amplitude of 20 dB, but its bases (48 and 56 kHz) have an amplitude of 7 dB. Once peaks were established, their peak frequencies were classified into 2 dimensions (teal parentheses): Low (20-59 kHz) and High (60-100 kHz). USVs were further classified into groups depending on where there peaks were found (see text and Table 2). This diagram is a multi-frequency USV (MF) because it has greater than 3 peaks. The reported frequency for each USV is the 'Dominant frequency' defined as the frequency with the greatest amplitude within a single USV. kHz: kilohertz. dB: decibel. Ms: millisecond

Category	Definition
Number of USVs	A sound which contained a definable 'peak'
Total	Sum of all USVs
MF	Multiple peaks observed across all frequencies often at regularly spaced intervals
H2	two peak observed between 60-100 kHz
H1	A single peak between 60-100 kHz
L1H2	One peak between 20-59 kHZ, two peaks between 60-100kHz
L2	Two peak observed between 20-59kHz
L1H1	One peak between 20-59 kHZ, one peak between 60-100kHz
L1	A single peak observed between 20-59 kHz.
Call Amplitude (dB)	The loudness of the sounds in decibles
Call Amp-Ave	Average amplitude for the 'dominant' frequencies
Call Amp-Max	The greatest amplitude recorded.
Call Duration (ms)	The length of time that USVs were continuous
Call Dur-Ave	The average duration of sustained USVs
Call Dur-Max	The single longest sustained USV
Call Dur-Total	The sum of time that USVs were recorded
Call Frequency	The frequency (kHz) with the greatest amplitude within a call (i.e. the frequency at the highest point of the peak)
Average Frequency	The average of all call frequencies
Call-Freq-max	The single highest-pitched frequency from any USV during the session.
Bout Characteristics	A temporal grouping of USVs separated by 1 s from other groups
Number of Bouts	The sum of bouts
Calls/Bout-Ave	The average number of calls within a bout
Calls/Bout-Max	The single highest number of USVs within a bout

Table 2. Definitions for USV analysis.

These user-defined classifications describe characteristics of the litter produced sounds. USV: ultrasonic vocalization. kHz: kilohertz. dB: decibel. Ms: millisecond. AVE: average per recording. MAX: maximum per recording.

Each time point (binned 30 ms of recording) was classified as noise or as an USV. USVs were defined as ultrasounds that had a 'peak' in frequency between 20-100 kHz. Any 'peaks' whose amplitude did not reach 15 dB were excluded from analysis. A 'peak' was defined as a local maximum point with two 'base' frequencies with a bandwidth of 5 kHz in either direction. The average amplitude of these 'base' frequencies needed to be on average 5 dB less than the dB of the 'peak' frequency. Frequencies between 20-59 kHz were defined as 'low frequency' while peaks found between 60-100 kHz were defined as 'high' frequency (see Figure 19).

Using these definitions, USVs were further classified by the frequencies of the peaks into categories (see Table 2): L1 (1 low frequency peak), L1H1 (1 low frequency peak/1 high frequency peak), L1H2 (1 low frequency peak /2 high frequency peaks), H1 (1 high frequency), H2 (2 high frequency peaks), and MF (multiple frequencies with peaks).

Other data collected using more temporally specific recording devices and analysis (McMurray 2011), has shown that typical calls from individual pups last between 50-80 ms. We therefore operationally defined 'calls' as temporally continuous USVs, with a minimum duration of 30 ms (See Figure 20). Using this definition, we could measure the average and maximum duration of 'calls'. It must be recognized that this operational definition of a 'call' may be a sound continuously produced by a single pup, or multiple pups emitting sound in close temporal order. Nonetheless, they represent continuous pup-produced USVs that the dams can hear.

Within each 'call' a 'dominant frequency' was measured, defined as the frequency with the greatest amplitude within the power spectrum of the 'call' (See Figure 20). This measurement was found for each call, and averaged together for each recording session. Additionally, a maximum dominant frequency for each recording session was defined as the highest frequency of all the 'dominant frequencies' from that recording. Amplitude of the 'dominant' frequency was also measured for each 'call'. Similar to the frequency measurement, amplitudes for each USV were averaged for each session, and maximum amplitude of the dominant frequency was determined for each session.

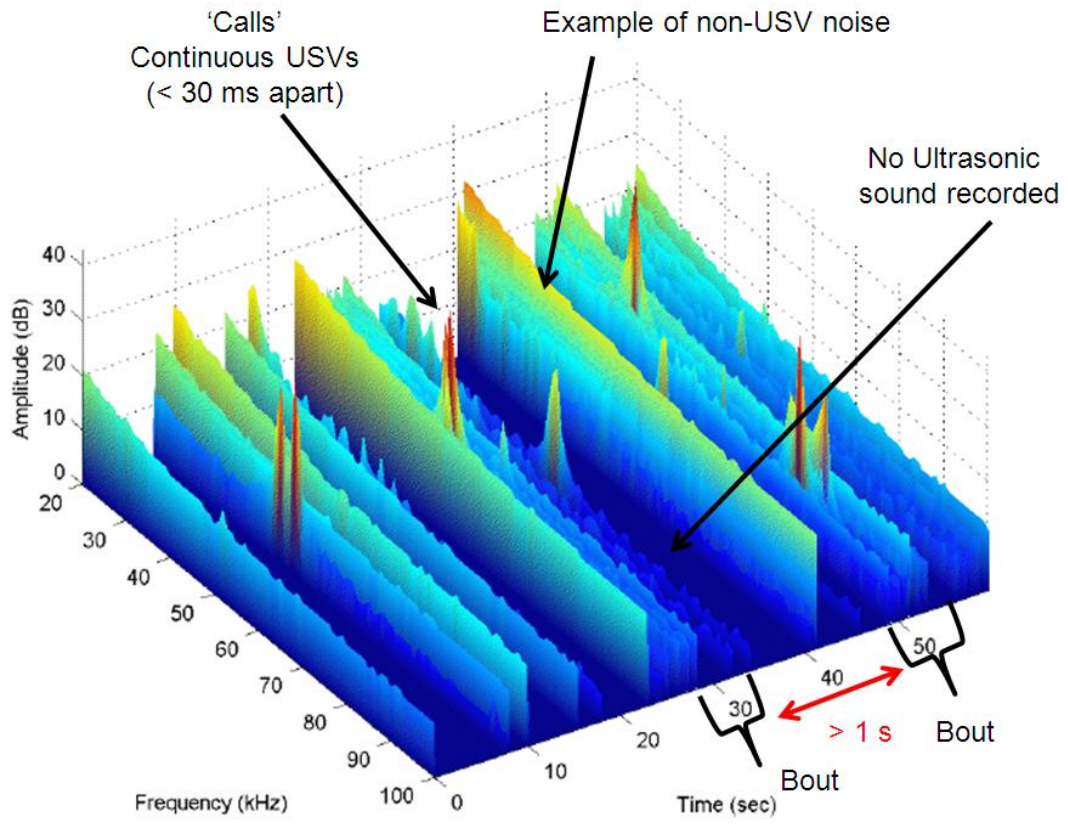

Figure 20. Representative image of USVs across time of session.

Frequency (kHz) is on the x –axis. Amplitude (dB) is on the y-axis. Time is represented on the z-axis. A 'Call' was defined as consecutive time points (30 ms) of USVs (black arrow at red and yellow peaks), but also includes USVs of a single time point for calculation purposes. The 'calls' arrow demonstrates an example of a 'call' with 2 time points and a duration of 60 ms. 'Calls' were counted individually if they were separated by more than 30 ms. A 'bout' (black parentheses) could contain several 'calls' if they were less than 1 second apart. 'Bouts' must be greater than 1 second apart (red arrow). This diagram also illustrates an example of the noise that is removed and periods of time when no ultrasonic noise was recorded.

It has been reported that rodent dams respond not only to the total number of calls or USVs from pups, but their temporal organization. Therefore the 'bouts' of calls were measured as well. A 'bout' was operationally defined as a set of calls, with less than 1 second of time between any individual call (see Figure 20). The number of bouts, and the number of calls per bout (average and maximum) was determined for each session.

A Student t-test was conducted to compare each of the above measures of the temporal and

spectral characteristics of the combined USVs in PNDs 1 and 3. On PND 5, a 1-way ANOVA was used to analyze group differences. As there were minimal treatment effects at any age, data were pooled to determine age-related differences using 1-way ANOVA and Bonferonni post-hoc analysis.

6.3.3 Experiment 3: Pup Urine Constituent Analysis

Urine Collection and Urinalysis:

Rat pups cannot autonomously urinate or defecate and require tactile stimulation to release these excretions through a reflex. Therefore, pup urine was collected by trained experimenters who gently held pups and stroked the anogenital region with a soft paintbrush (similar to licking they receive from a dam) to elicit excretion. When urine was excreted in the absence of feces, a pipette tip was used to collect the sample. Urine samples were collected separately for each sex and from at least 2 male and 2 female pups per litter per day. Following the behavioral test, urine samples were frozen and stored at -80° C until assays were performed.

Pup urine was thawed and tested with Seimens Multistix 10 SG Reagent strips for Urinanalysis (Tarrytown, NY) per manufacturers' instructions. These strips measure amounts in mg/dL unless otherwise noted: protein, nitrite, glucose, ketones, pH, specific gravity, bilirubin, urobilinogen, blood, and leukocytes (5-15 white blood cells/high power field).

Statistical Analysis

One-way ANOVAs were used to determine differences were between UN, CS-exposed, and CC-exposed urine. Since analyses showed no effect of CS treatment on PND 5, CS-exposed urine was excluded from a 3-way ANOVA on UN and CC-exposed urine samples. Three way ANOVAs (Treatment X Age X Sex) followed by hypothesis tests for specific differences were primarily used to determine differences between UN and CC-exposed pup urine characteristics on PNDs 1, 3, and 5.

6.3. RESULTS

6.3.1 Overall Gestational Data and Pup Growth:

All gestational and pup growth data is presented in tables in the Appendix E; Table 11 and 12. Although gestational length was the same across treatment groups, within the pup-provider (PP) dams, it was observed that CC-treated dams were more likely (χ^2 (1) = 24.838, $p \leq 0.001$) to start labor earlier compared to UN dams. CC-treated dams gained less weight than UN (F (1,37) = 4.909, $p \leq 0.05$) and CS-treated (F (1,37) = 5.533, $p \leq 0.05$) dams by the end of pregnancy. CC-treated dams weighed significantly less at the end of gestation (t = -3.937, $p \leq 0.05$). However, CC-treated dams gained more weight during the first postpartum week compared to UN dams (t = 2.827, $p \leq 0.001$).

Since there were no initial differences between pups born to test dams and pup-provider dams, and a 1-way ANOVA suggested no difference between UN and CS-exposed pups, their data were pooled for analysis. There was a small, but significant, effect on PND 1, such that CC-exposed pups weighed less than controls (t =-3.076, $p \leq 0.001$). There were no differences in sex ratio, litter weight, culled litter weight, or weight gain during their first neonatal week (see Appendix F; Table 12).

6.3.2 Experiment 2: Litter Ultrasonic Vocalizations (USVs)

Body Temperature

There were no treatment effects on body temperature measures. All data can be found in Table 10 in the Appendix F. PND 1 pups were significantly cooler when taken from the nest compared to PND 3 (F (1, 174) = 22.749, $p \leq 0.001$) and PND 5 (F (1, 174) = 48.062, $p \leq 0.001$). PND 3 pups were cooler than PND 5 pups (F (1, 174) = 5.760, $p \leq 0.05$). When the USV recording session began PND 1 pups were cooler than pups on PND 3 (F (1, 197) = 152.060, $p \leq 0.001$) or PND 5 (F (1, 197) = 267.221, $p \leq 0.001$). Interestingly, PND 1 pups gained more heat

during the test compared to pups at PND 3 (F (1, 195) = 18.886, $p \leq 0.01$) and PND 5 (F (1, 195) = 5.526, $p \leq 0.05$), which showed no change.

Total Ultrasonic Group Measures

Since it is currently unclear whether dam response is dependent on USVs or the summation of all ultrasonic sounds in the environment, we analyzed the acoustic characteristics of recordings prior to filtering. There were no differences in the total number of ultrasonic sounds across treatment conditions, however a main effect of age indicated such that PND 1 pups had a greater number of sounds in the 20-59 kHz frequency range compared ton pups at PND 3 (F (1, 202) = 16.269, $p \leq 0.001$) and PND 5 (F (1, 202) =22.344, $p \leq 0.001$). All PND 1 pups also produced a significantly greater number of (60-100 kHz) sounds compared to PND 3 (F (1, 202) =10.499, $p \leq 0.001$) and PND 5 pups (F (1, 202) = 19.904, $p \leq 0.001$).

Ultrasonic vocalizations and Pup Calls

The acoustic characteristics of USVs at each age for each group are listed in the Appendix F, Table 13. There were no significant treatment effects observed in any of the USVs and 'call' characteristics. However, there was a main effect of age, on several measures of calls within the groups (see Figure 21). PND1 pups had fewer calls (F (1,273) = 5.859, $p \leq 0.05$), fewer calls per bout (F (1,276) = 15.597, $p \leq 0.05$), and more bouts (F (1,279) = 12.592, $p \leq 0.05$) than PND 3 pups. These calls had lower amplitude (F (1,278) = 32.446, $p \leq 0.001$), shorter average duration (F (1,271) = 3.961, $p \leq 0.05$), shorter total duration (F (1,271) = 6.401, $p \leq 0.05$), lower peak frequencies (F (1,278) = 19.381, $p \leq 0.001$). Additionally, PND 3 pups differed from PND 5 with fewer calls (F (1,273) = 4.351, $p \leq 0.05$), lower amplitude (F (1,278) = 20.885, $p \leq 0.001$), shorter average duration (F (1,271) = 11.120, $p \leq 0.01$), shorter total duration (F (1,271) = 3.800, $p \leq 0.05$), lower peak frequencies (F (1,278) =3 3.762, $p \leq 0.001$).

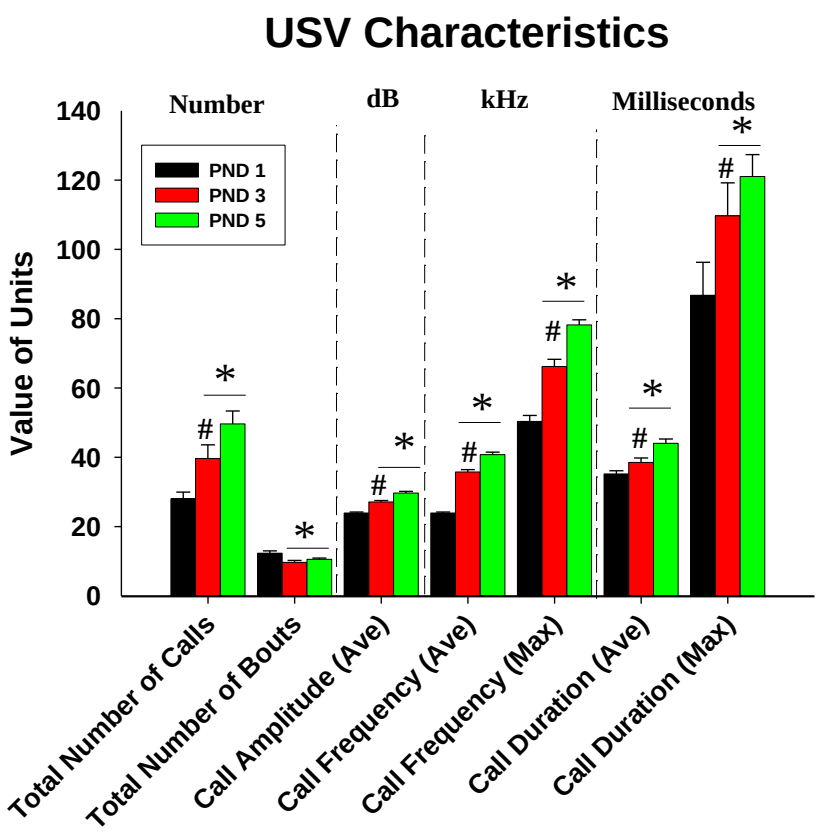

Figure 21 USV Characteristics Across Age.

Data presented as means ± SEM per age group. Value of units is represented on y axis. Units are listed across the top and separated by dotted vertical lines. The quality of calls changed in a variety of dimensions as the pups aged. There was a main effect of age in the total number of calls, call amplitude, average and max call frequency, average and max duration of calls (represented as lines with asterisks). PND 1 pups have more bouts compared to older pups. (*= differs from PND 1, $p \leq 0.05$; # = differs from PND 5, $p \leq 0.05$.) dB: decibel. kHz: kilohertz

6.3.3 Experiment 3: Urinalysis

All relevant results can be found in Figure 22. Few sex effects were found at any age; therefore, male and female data were pooled for presentation.

Glucose: The only urine samples with any detectable glucose were CC-exposed pups at PND 1, CC-exposed pups were more likely to have glucose in their urine compared to UN pups at PND 1 (χ^2 (1) = 19.926, $p \leq 0.001$). However, only 30.5% of litters showed this phenotype.

Bilirubin: Similarly, bilirubin was only present in 26.6% of urine samples on PND 1, with

no group or sex differences.

Ketones: Only a small proportion of urine samples had any detectable ketones. On PND 1, only 34.5% of pups had detectable levels, however, CC-exposed pups were more likely to have ketones (50.9%) compared to UN pups (16.9%; χ^2 (1) = 13.996, $p \leq 0.001$). A 3-way ANOVA revealed a treatment X age interaction (F (2, 368) = 5.039, $p \leq 0.01$). Post hoc analyses indicated that on PND 1, CC-exposed pups had higher levels of ketones than UN pups (F (1, 368) = 14.465, $p \leq 0.001$). Since ketones may interfere with the detection of glucose, a linear regression was run to show that ketone levels were not associated with glucose levels (F (1, 54) = 0.697, p = 0.47) in pup urine on PND 1.

Blood: A 3-way ANOVA revealed no interactions effects but indicated that PND 1 pups had much higher levels of blood in the urine compared to PND 3 (F (1, 360) = 274.521,$p \leq 0.001$) and PND 5 (F (1, 360) = 235.010, $p \leq 0.001$). PND 5 urine showed a small but significant increase in blood amounts (F (1, 360) = 3.383, $p \leq 0.05$) There were no significant between group treatment effects at any age.

Protein: A 3-way ANOVA indicated a treatment X age interaction (F (2, 340) = 4.142, $p \leq 0.05$), and post hoc tests found that CC-exposed pups had higher protein levels compared to UN pups on PND 1 (F (1, 340) = 6.780,$p \leq 0.01$). UN pups had higher, protein levels on PND 1 than on PND 3 (F (1, 340) = 18.353, $p \leq 0.001$) or PND 5 (F (1, 340) = 35.990, $p \leq 0.001$). Within group differences for CC-exposed pups can be seen in Figure 22.

pH: A 3-way ANOVA revealed that PND 1 pups have significantly higher pH values compared to PND 3 (F (1, 360) = 110.848, $p \leq 0.001$) and PND 5 (F (1, 360) = 102.696, $p \leq 0.001$) pup urine samples, regardless of treatment or sex.

Leukocytes: 3-way ANOVA revealed a treatment X age interaction (F (2, 328) = 3.649, $p < 0.05$). Post hoc tests found that on PND 1, CC-exposed pup urine had fewer leukocytes than UN pups (F (1, 328) = 4.060, $p \leq 0.05$) and compared to CC-exposed pups at PND 3 pups (F (1, 328)

= 9.491, p ≤0.01). UN pups had significantly fewer leukocytes on PND 5 compared to PND 3 (F (1, 328) = 4.981, p ≤0.05). Only (19.2%), (50.9%) and (21.8%) of samples had detectable levels of leukocytes on PNDs 1, 3 and 5 respectively for all groups. Although high levels of glucose can increase the likelihood of a false positive for leukocytes, glucose was only observed in CC-exposed pups on PND 1 indicating that glucose did not interfere with the test as they had few.

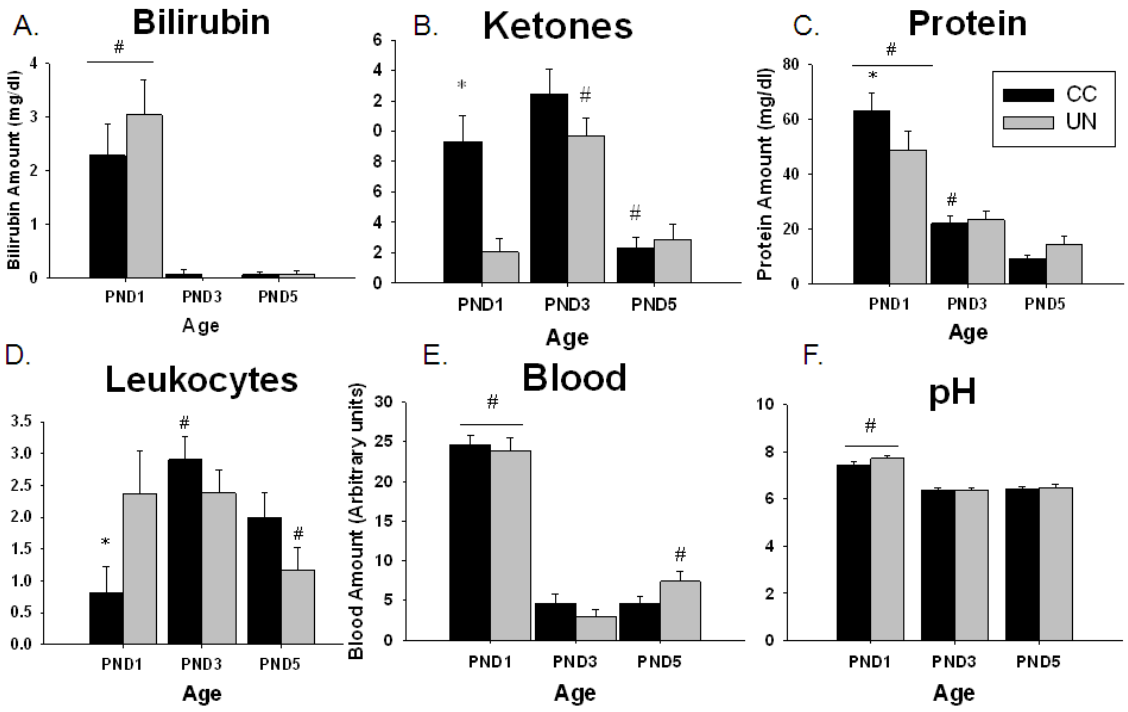

Figure 22 Urinalysis Results Across the first Neonatal Week.

All data represented as means ± SEM of treatment groups by age. A-C. The amount of item was determined in mg/ml. D.) Determined in approximate white blood cells/ high powered field E) Amount of blood were given arbitrary numerical values to correspond with relative amounts determined from test. Significant within group age effects are represented by #= p < 0.05. Within age treatment effects are represented by asterisks (* = p< 0.05).

6.4. DISCUSSION

CC exposure does not significantly alter the characteristics of litter USVs that were measured in this study, although other spectral characteristics may be altered and those qualities may be the underlying causes for dam preference for pups in the retrieval task. The lack of many differences in pup USVs may not be surprising, given that the single published study has

investigated the effect of PC exposure on early infant mouse USVs, found small strain-specific decreases in the number and frequency of USVs produced by pups immediately removed from the nest and placed on a chilled surface (Hahn et al. 2000), indicating that genetic background was important for observing a teratological effect. Recent evidence suggests that individual isolated UN pups kept at constant temperature are more likely to emit USVs compared to CC-exposed pups at PND 3, but not PND 5 (McMurray 2011). Future work using more sophisticated temporal recordings of litter USVs could answer this question.

Although the presented data agreed with previous literature, indicating that the number of USVs emitted by pups increases with age during the first postnatal week (Branchi et al. 2001), it is possible that the experimental procedure may have impacted our results which indicated no group differences. Studies of rodent pup USVs vary widely in the experimental procedures with differences in separation time, temperature maintained, and isolation techniques. The number of USVs emitted can be greatly affected by isolation length and separation from littermates, such that contact with littermates can reduce USVs (Shair et al. 2003), thus it is possible that pups reacted to their reintroduction to the litter (see Methods) with fewer USVs than had they remained separated. This conclusion is supported by previous work which demonstrated that littermate contact could equally decrease separation-induced USVs in both control and PC-exposed pups (Goodwin et al. 1993).

When interpreting USV measurements, it is important to note that recordings were taken from a litter of mixed sex pups rather than individuals. It is well established that male and female pups emit USVs with different qualities in a dynamic way throughout development (Hahn & Lavooy 2005). This experimental procedure and recording technique precludes the interpretation that any detected USVs were uniform across the litter. Although there is a loss of specificity for individual pup calls, we believe that multiple pup calls emitted simultaneously was more closely related to the natural litter environment of dams. That pups were piled in the cups keeping them warm may also have affected calls as colder pups are more likely to call. Future studies should

investigate USVs occurring in nest of undisturbed dams and pups. Current ongoing analysis is beginning to investigate how USVs may change when dams are introduced to a cage and pups can recognize her presence.

Our results reporting slow weight gain in dams and reduced pup weight at PND 1 agree with some previous studies (Johns et al. 2005, Johns et al. 2007, McMurray et al. 2008), but not others using the same treatment paradigm (Johns & Noonan 1995,Johns et al. 1994, Lubin et al. 2001), suggesting that this effect may be sensitive to environmental changes or experimental error and thus must be further characterized before firm conclusion can be made. The impact of PC exposure in human infants on birth weight and growth has been reviewed several times (Nassogne et al. 1998, Frank et al. 2001), concluding that heavy cocaine use typically results in low birth weight, after confounding variables are taken into account, such as gestational age, maternal age, socioeconomic status, concomitant drug use, and prenatal medical care are controlled for. However, some reports show no difference in birth weight or head circumference in children (Zuckerman et al. 1989, Hurt et al. 2008). Infants exposed to cocaine use, especially heavy cocaine use were more likely to be categorized into a group with high risk for later medical and behavioral problems, including early birth, low birth weight, and abnormal brain ultrasounds (Liu et al. 2010). It has been suggested that low birth weight may be caused by anorectic effects of cocaine use, poor maternal nutrition, fetal vasoconstriction and/or faster fetal metabolic rates. This suggests that a comparison to our CS-exposed groups, which are food restricted during the first week, may be a more proper control and future studies will take this into account.

Low birth weight may be indicative other physiological problems, since urine composition is determined by the kidney, our results suggest that kidney function may be abnormal in some of the pups since there were many detectable differences between UN and CC-exposed pup urine, especially on PND 1, indicative of such issues. Cocaine during early pregnancy in rodents can cause delayed development of the kidney, although this effect is corrected by weaning, (Hunter et al. 1995). Human data supports deficits in kidney formation and function as well, immediately

after birth in PC-exposed infants (Ho et al. 1994). Fetal vasoconstriction may play a role in the development of the kidneys and liver (Sanders et al. 2004), and it a well-established contributor to the effects of PC exposure (Lester & Padbury 2009). Glucose in the urine (glucosuria) is a symptom of glucose in the blood, and hyperglycemia is a symptom of troubled kidney function as well as many other potential physiological problems. Cocaine has been shown to increase fetal plasma glucose levels (Owiny et al. 1991), and since cocaine and its metabolites are still observed in urine of PND 5 CC-exposed pups (McMurray 2011), it is possible that these pups may be hyperglycemic, and this should be tested in future studies. Alternatively, glucosuria is more common in premature and low birth weight human infants (Falcao et al. 1999), traits that we see some signs of in our CC-exposed pups, suggesting that circulating cocaine need not play a role. Although, glucose it not thought to have a strong odor, hyperglycemia is believed to change urine and body odor (Dalton et al. 2004), and dams may respond differently to these odor differences (see Chapter 5) or to the taste in urine when licking pups.

We observed that in pups that had detectable bilirubin, CC-exposed pups had less in their urine, although this difference was not significant, it hints at a potential difference in liver function or hemolysis. Hyperbilirubnemia, presents in approximately 60% of newborns for the first week of life in human infants. Although, the majority of cases resolve after the first week, this symptom can be a symptom of hemolytic disease, metabolic and endocrine disorders, anatomic abnormalities of the liver, and infections (Cohen et al. 2010). In the clinical population, risk factors include prematurity, male sex, and infrequent feedings. PC exposure in human infants resulted in deceased urinary bilirubin, believed to be a result of increased glutathione-S-transferase and bilirubin uridine diphosphate-glucuronosyl transferase activity (Wennberg et al. 1994), however other reports show no difference in hyperbilirubnemia (Scafidi et al. 1996). Bilirubin is a particularly pungent chemical, and thus may play a role in olfactory responses from dams (see Chapter 5).

Protein in the urine can be the result of either nephrological or urinary disorders, although

high levels of blood in the urine can give falsely high readings of protein. This may be the case in urine tested at PND 1, when many of the samples had very large amounts of blood. It seems that a decrease from birth through the first week of life is typical as levels normalize to adult values (0.1-50 mg/ml) in all prenatal treatment conditions. It is currently unclear what proteins were increased in the urine, an issue that should be further pursued. Proteins may play an important role in olfactory preference behavior, since specific proteins (MHC III markers) in urine are typically used by rodents to identify one another (Cotton 2007).

Urinary ketones are a symptom of ketoacidosis, which is commonly observed in diabetic patients, along with hyperglycemia as a result of insulin insensitivity. Ketoacidosis is a symptom dehydration and malnourishment. PC-exposed babies have been shown to have lower fat deposits which may be a result of faster metabolic rates (Nassogne et al. 1998, Frank et al. 1990). Fetal body fat is also reduced in rodents following PC exposure (Church et al. 1995). Ketones are known to give urine a sweet or fruity odor (Simerville et al. 2005), and thus may play an important role in olfactory investigation or preference.

Urinary pH is a signal of serum pH and should remain primarily neutral to slightly acidic. Our results confirm that pup urine is within normal ranges although the higher pH on PND 1 may be caused by metabolic differences experienced immediately after birth. pH can affect odorants volatility and water solubility which can impact the odorants path through the nose to the olfactory epilthelia (Schoenfeld & Cleland 2005), suggesting that PND 1 urine constituents may have greater potential to affect the olfactory receptor neurons, potentially enhancing their behavioral salience (see Chapter 5).

The majority of pups show hematuria on PND 1, with decreasing amounts with age. Hematuria can be caused by glomerular or other structural disruptions in the kidney or another common cause in human infants is a urinary tract infection, both factors that have been associated with PC exposure (Gottbrath-Flaherty et al. 1995). Although the pups were observed to have been licked and ‘cleaned’ by the dam, it is possible that blood remained in the skin, creating an artifact

on PND 1. Blood odor typically causes a fear response in adult rats (Hornbuckle & Beall 1974, Stevens & Gerzog-Thomas 1977), although this seems to be diminished immediately postpartum.

There were no differences in specific gravity due to prenatal treatment, age or sex, we can conclude that pups were all equally hydrated and that this measure likely does not play an important role in olfactory preference. The compound measuring leukocyte activity is produced by neutrophils in response to infection, and are commonly associated with urinary tract infections (UTIs) (Simerville et al. 2005). PC-exposed infants are more likely to have UTIs compared to non-exposed infants (Gottbrath-Flaherty et al. 1995), however our results suggest CC-exposed rodents have fewer leukocytes, suggesting perhaps a weakened immune response. It has recently been shown that rats avoid the bedding (odor) of other infected rats (Arakawa et al. 2010), suggesting that if PC-exposed infants are more likely to get sick, this may impact olfactory preference.

Taken together, the urine constituent differences in CC-exposed pups compared to UN pups indicate one possible mechanism for olfactory behavioral preferences whether through aversion to the odor (CC-treated dams) or attraction to what might represent a sick pup in need of maternal care (UN dams). In any case these attributes both suggest physiological abnormalities in the early postnatal period for CC-exposed pups and also are translational to the human PC-exposed infant. Comparisons to other potentially malnourished groups would clarify the contribution of PC exposure. The great degree of variability observed in results from PND 1 may be a result of the timing of urine collection. Although all pups had observable milk bands (i.e. they had nursed at least once) prior to urine collection, the amount of time between their birth and urine collection could vary as much as 12 hours. It is currently unclear how urinary results may differ from individual pups within the same litter, and the contribution of urinary cocaine to the likelihood of other urinary changes. Although this would be an interesting future project, feasibility would require a technique that can measure all the constituents in urine from smaller volumes. Alternatively, the observed differences in PND 1 pup urine may be an artifact of maternal

nutrition and health status at the time of birth, as cocaine may be affecting the physiology of the dam as well. Future studies could investigate more closely the health of dams immediately prior to parturition to determine the possible maternal contribution to observed effects in PC offspring.

CHAPTER 7. GENERAL DISCUSSION

7.1. OVERALL CONCLUSIONS

Our goals were to examine how gestational cocaine treatment affects rodent dam choices for specific kinds of litters and relevant pup-produced cues and to examine several possible sources of underlying mechanisms that might impact the preference for and maternal behavior towards pups (or their cues). Choices of physiological mechanisms in dams were selected based on previous studies in the literature highlighting the endocrine factors (CORT, OT) influencing maternal response and behavior and choices of pup-produced stimuli were likewise known elicitors of maternal care. Our findings were relevant to both past studies and future directions in the area of maternal-infant interactions with an emphasis on any translational measures particular to both human and animal species.

These studies, along with many previous studies of maternal-infant interactions following gestational drug use or treatment, have necessarily focused on relatively narrow ranges of behaviors in temporally restricted tests. The present findings, along with past reports, highlight the many complex variables involved in mother-infant interactions and the rapidly changing biological environment of both mother and infant that drives many of these variables over the early postpartum period.

7.1.1 Pup-produced Cues: Dynamic Relative Importance to Dam Choice

A model of the relative contributions of different environmental stimuli and maternal endocrine changes to preference choice and MB across the first postpartum week is presented in Figure 22. CC-exposure appeared to have minimal effects on litter-produced USVs, and these

USVs did not directly correlate to maternal retrieval preference behavior at any day. The overall lack of retrieval latency effects may be a result of the fact that there was such variability in the USV production from different litters, so few dams experienced the hypothesized differences in number of pup USVs. Only approximately 50% of dams were exposed to pups who were calling at significantly different rates which reduces the power of such a measure. Even so, within a specific test session, when USVs from litters were compared such that if one group was noted to have more than double the number of USVs than another group, we could compare performance but no clear relationship was observed between the number of USVs and retrieval preference on any test day in those cases. Given the group litter variability, it would be preferable to have prerecorded litter calls that were known to differ on specific characteristics played back to dams for retrieval preference (perhaps in a CPP chamber) to compare dam groups for a controlled stimulus presentation. These studies are ongoing with single pup cries and perhaps will be used in future tests.

The urine constituent group differences were especially interesting given the described data that suggest physiological distress of a proportion of young infants. These effects are not observed in all CC-exposed litters, which may be an underlying cause for variability in response to CC-exposed pups as a group. As olfactory cues are highly important for this species, even slight differences could have a large impact on maternal response, whether curiosity, or in the case of CC-treated dams, perhaps aversion to the CC-exposed urine odors. Though our technology limitations prevented us from detecting additional differences that might be relevant to pup response (OT) and maternal response (dodecyl prioprionate), there were preferences by dams on PND 1 that so many tests have shown that CC-exposed pups are neglected more by many rat dams (Johns et al. 2005, Johns et al. 1994).

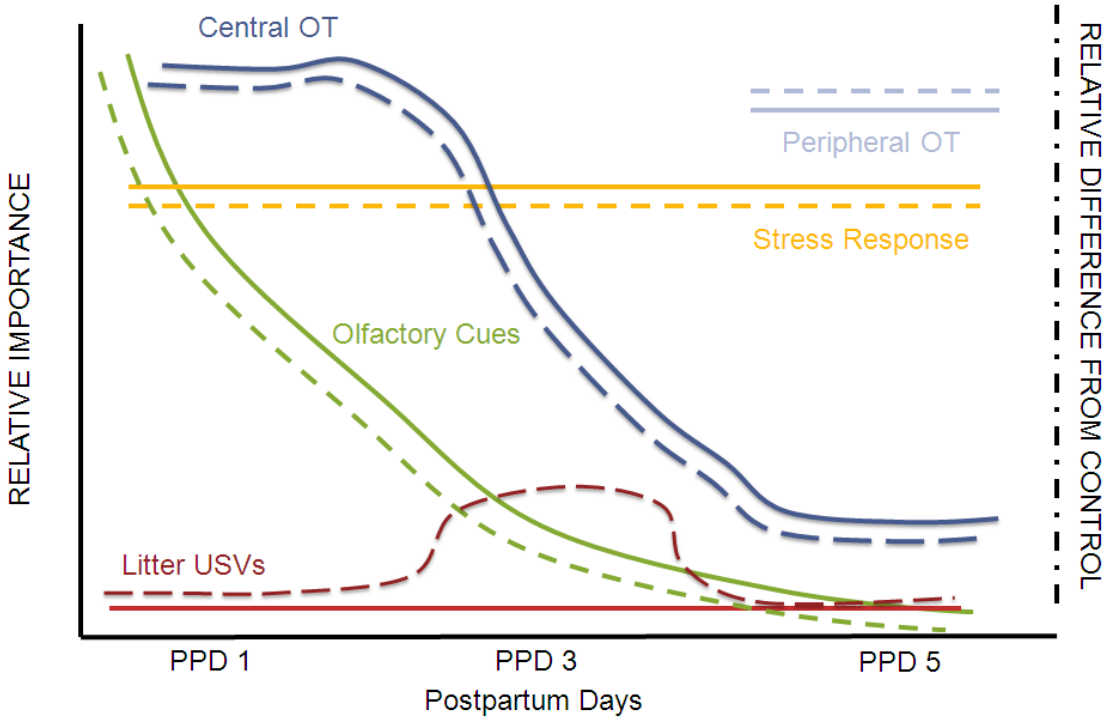

Figure 23. Model for the Relative Impact of Postpartum Factors on MB.

Pup-produced cues develop across the postnatal period, simultaneously these cues change in relevance to dams. In this model, relative importance to affecting MB is indicated by the left y axis (solid lines). Relative difference of CC-exposed animals from UN animals is represented in right y axis (dotted lines). Pup-produced olfactory cues (green lines) differ significantly on PND 1 and seem to diminish in importance across the postpartum period. Litter USVs (red lines) do not differ dramatically from controls and dams do not alter behavior based on litter USVs. CC-treated dams have disruptions in affective behaviors (anxiety/stress coping; orange lines) can drastically affect MB across the postpartum. Central OT regulation (dark purple lines) is more affected and more critical for MB in the early postpartum compared to later postpartum days. Peripheral OT (pale purple lines) differs greatly among treatment groups and likely affects stress and MB.

7.1.2 Transactional Maternal-Infant Relationship Development

Our results indicated that CC-exposed pups could elicit preferential retrieval by PND 5, suggesting some enhanced eliciting of attention had developed by this age. A similar effect was observed in human studies, indicating that although PC-exposed infants behaved differently in a social-interactions task at 6-months, this difference in behavior typically elicited more caregiver attention during the laboratory task (Lewis et al. 2009). Perhaps, given previous early experience, with neglect following attempts to elicit care, these infants have adopted a different strategy. Indeed, maternal responsiveness in rodents has been shown to contribute to later USV production

by pups, a known elicitor of retrieval (D'Amato et al. 2005). An intriguing study to perform, would investigate these behaviors in dams, with pups which have been cross-fostered to determine the effects of previous maternal neglect on CC-exposed pups.

It has recently been shown that deficits in maternal care observed in cocaine-abusing mothers at 13 months postpartum can be attributed to a disrupted transactional relationship with their infants. This study showed that decreased maternal sensitivity at 1 month postpartum led to increased hyper-reactivity in infants at 7 months, which led to increased passive parenting behaviors in their mothers (Eiden et al. 2011). This report highlights how initial behaviors of mothers with their infants can immediately impact infant behavior, and that these changes in infant behavior continue to influence maternal care received. Evidence suggests that cocaine-using mothers do not differ in mother-fetus attachment scores compared to non-drug using women (Shieh & Kravitz 2006), and strengthens the hypothesis that the behavioral interaction between mother and child are critically interdependent on behavior from infant as well as the mother.

7.1.3 Cocaine-Induced Alterations in Postpartum Endocrine Signaling

Another conclusion that can be drawn from these studies is that gestational cocaine exposure affects OT signaling in a context-specific fashion. CC-treated dams exhibit lower plasma OT levels following pup-interaction retrieval test compared to both UN and CS-treated dams. Interestingly however, following the stressful and non-pup related exposure to the forced swim tank, CC-treated dams exhibited much higher plasma OT compared to UN dams. This would suggest that there is not major damage to the integrity of the OT system (hypothalamus, OT cells or OT release mechanisms), but rather that changes may be occurring in the regulatory aspects of OT release (Figure 24).

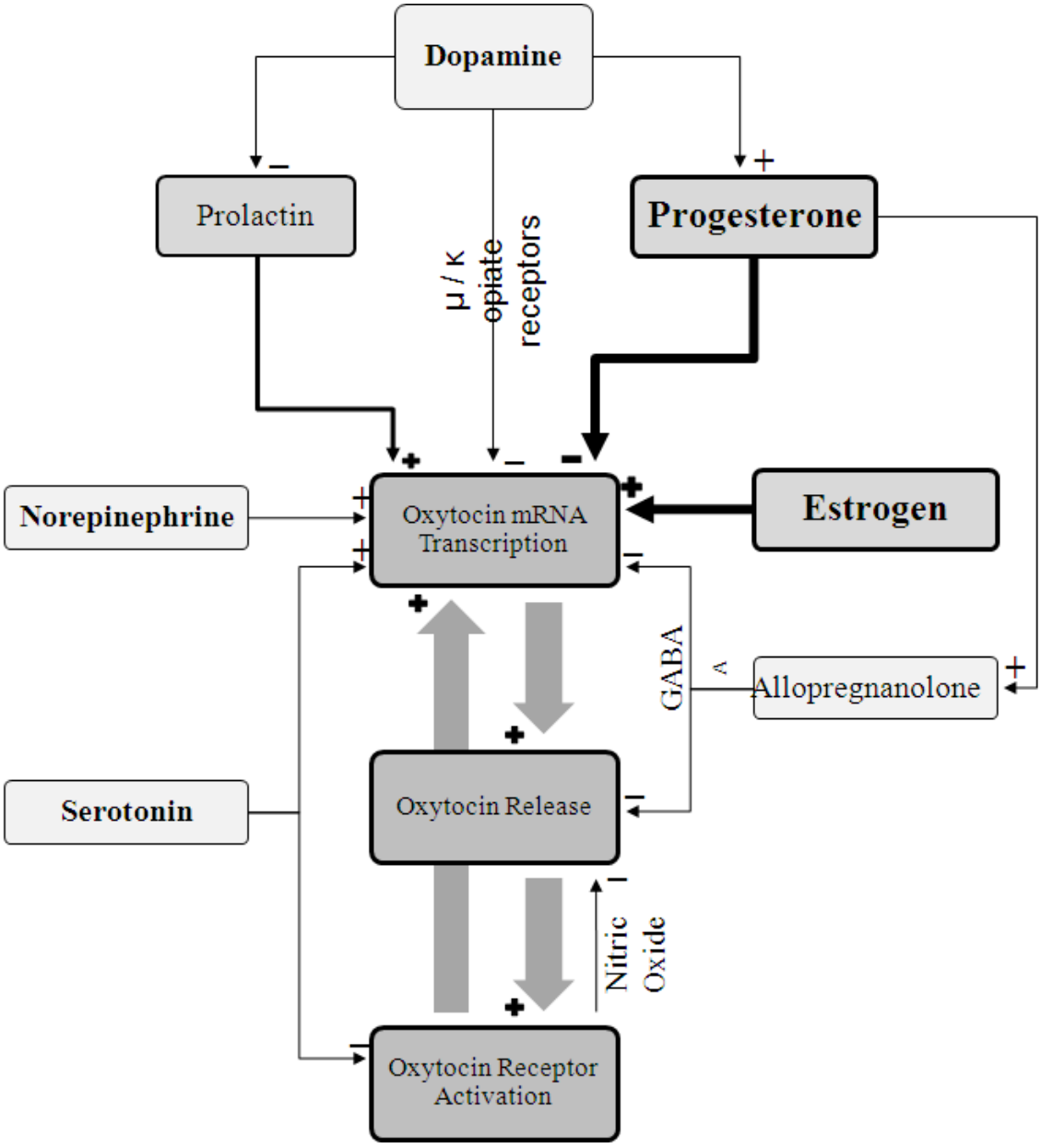

Figure 24. Regulation of Oxytocin production.

Oxytocin mRNA production is increased by estrogen, prolactin, norepinephrine, serotonin and OT receptor activation. mRNA production is decreased by progesterone, opoids, GABAA receptors. Cocaine can disrupt many of these signaling systems, however, whether one or many disruptions are necessary remains unclear. Figure adapted from (McMurray et al. 2008)

It was previously shown that acute cocaine can impact OT release in a brain regional- and PPD specific fashion (Elliott et al. 2001). Following immediate removal from the nest, CC-treated dams, compared to CS-treated dams, had lower OT levels in the MPOA, but not VTA, AMY, or HIPP; however, by PND 2, continued reductions are observed in the MPOA and significant decreases were observed in VTA and HIPP (Johns et al. 1997). The current results suggest that following maternal retrieval testing, CC treatment did not affect central OT levels in the hypothalamus, AMY, VTA or NAc compared to UN dams but it did alter AMY and hypothalamic OT levels in opposite directions following the stressful FST where no pups were involved. The previous studies examined OT within the first 3 days following pup delivery at a time when OT is thought to be most critical to MB onset whereas the present study looked at levels at a later time point (PPD 5) which is correlated with fewer disruptive effects on MB in CC-treated dams (Johns et al. 2005). It may be that cocaine treatment has its greatest central effects in the very early PP period but has continuing stress related effects on peripheral OT that could continue to affect the mother's behavior via indirect non-adaptive stress effects.

There are many mechanisms through which CC treatment could be altering OT release (see Figure 24). Studies investigating the mechanisms of CC treatment have suggested that combined DA/5-HT transporters blockade are important contributing mechanisms for altered central OT release observed in CC-treated dams in the postpartum period (Johns et al. 2005). However, the direct mechanistic changes (changes in DAT/SERT, receptors, etc) that occur to control release have not yet been determined. OT, through interactions with the dorsal motor nucleus of the vagus, can produce 'immobility without fear' but this may be more pronounced in females compared to males which have a different response pattern to stress (Carter 2007). We did not observe an increase in hypothalamic OT in response to FST exposure, in fact in CC-treated dams we observed a reduction compared to dams who were taken from the nest. This may be a result of a massive release of the available OT into plasma, such as that observed in males following the FST (Wotjak et al. 1998, Engelmann et al. 2006, Ebner et al. 2005).

CORT hormone levels maintain the pulsitude rhythmicity during lactation that is seen during estrous cycling in females rats, though the diurnal peaking is diminished (Atkinson & Waddell 1995, Lightman et al. 2001). OT levels are also regulated in a circadian rhythm (Bertram et al. 2010), although with a slower cycling pattern than CORT, but are more dependent on lactation schedule during the postpartum. These different rhythms lead to unsynchronized OT and CORT levels in typical dams and in humans during lactation (Handlin et al. 2009). CC-treated dams exhibited a greater coordination between plasma CORT and OT, and a blunted circadian CORT rhythm compared to UN dams. Cocaine may be disrupting circadian signaling through affecting clock genes throughout the central nervous system or though melatonin signaling (Perreau-Lenz & Spanagel 2008). The role of melatonin signaling may be an especially interesting avenue of future research since it has shown it to modulate stress-induced OT release (Juszczak 1998, Juszczak & Boczek-Leszczyk 2010). Given the importance of circulating hormones to anxiety, depression and stress responsiveness, this area deserves future study (Brunton & Russell 2008).

7.1.4 Model for Gestational Cocaine Abuse on Maternal-Infant Interactions

Taken together, these studies indicate that gestational treatment with cocaine can cause a variety of behavioral and neurobiological effects in mothers and offspring which can interact to disrupt maternal-infant interactions. As depicted in Figure 25, I posit that a number of postpartum variables are altered by gestational cocaine treatment and that each of them have been independently shown to contribute to maternal-infant interactions in drug-free scenarios. Therefore, they each represent a path through which cocaine could disrupt some aspect of maternal care; however, it remains unclear which of these variables is most important for specific deficits in care (i.e. retrieval, nursing, grooming) and this needs to be further explored; but also reminds us that the maternal system is redundant and highly malleable. Likewise, the infant, from even a very young age, appears to be at once very resilient and can survive many adverse

conditions such as neglect; but as we are now discovering, remains in many ways more vulnerable than non-traumatized offspring and only under conditions of stress do these detrimental and enduring effects appear. As such, there is likely not one stimulus that can totally disrupt care, and these studies must consider this aspect. Another important concept that these studies support is that of the transactional model of parent-child interactions. As depicted, altered infant physiology and behavior can be conceived as a negative endpoint of itself, unfortunately, altered infant phenotype may negatively affect maternal care, initiating a vicious cycle. However, the situation may not be as bleak as it seems. This model also points to several areas where intervention is not only possible in clinical populations it is feasible as well.

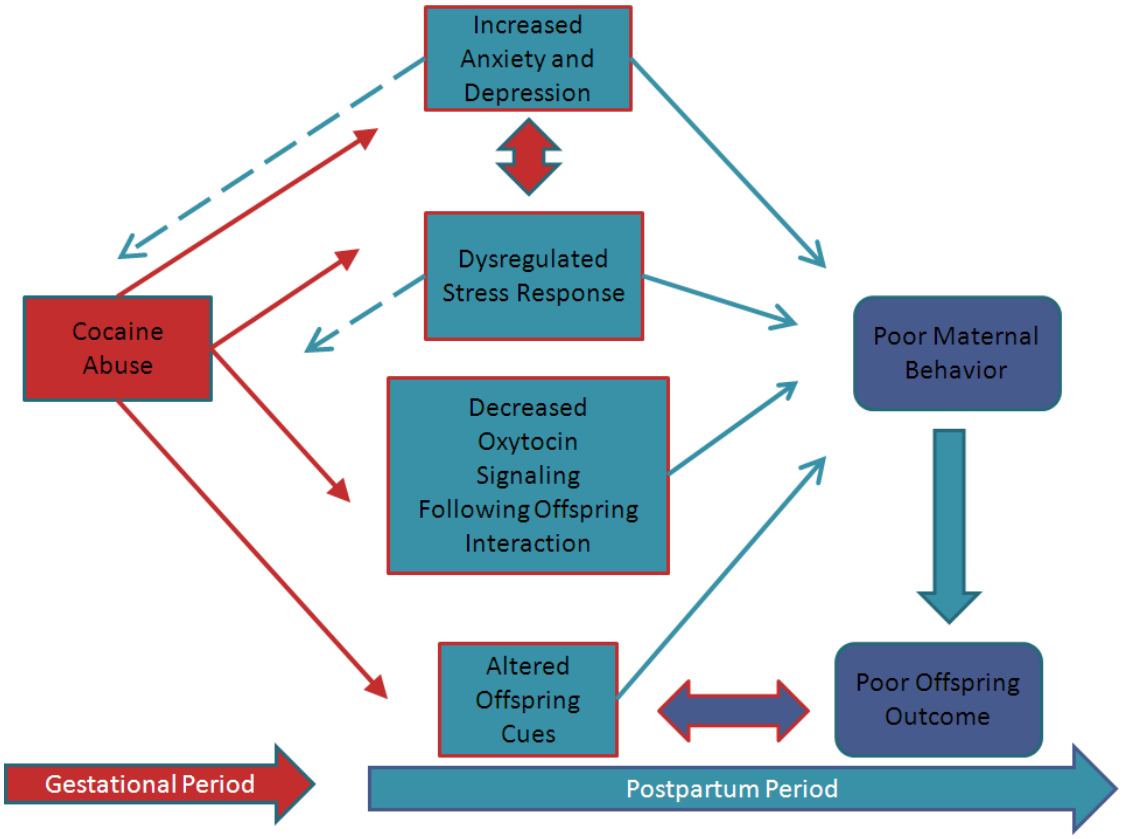

Figure 25. Model for Gestational Cocaine Abuse on Maternal-Infant Interactions.

Cocaine abuse (red box) during pregnancy (Gestational Period; red block arrow) can lead to a variety of behavioral and neurological effects in the postpartum period (teal boxes). Many of these factors are inter-related (red double-headed arrow), for example anxiety and depression are greatly modified by stress response. These in turn may increase the likelihood of continued or relapse of drug use in mothers (teal dashed arrows). Increases in cocaine-induced effects (teal boxes) in the postpartum period have been shown to contribute to poor MB (purple box). Poor MB can lead to poor offspring outcomes (both psychologically and physiologically) resulting in greater differences in offspring-produced cues. These leads to a cycle of perpetuating disrupted neglect, indicating that early intervention is key.

7.2. FUTURE DIRECTIONS

7.2.1 Cocaine-Induced Deficits in Maternal Motivation

Although retrieval is interpreted as an appetitive motivated behavior (Numan & Stolzenberg 2009), there are other behavioral tests that could measure the motivational value of pups to dams (Berridge 2004). For example, CPP has been used to determine whether pup availability can elicit

a ‘reward-seeking’ behavioral phenotype (Wansaw et al. 2008). Alternatively, intracranial self-stimulation studies could be used to assess differences in motivational circuitry not dependent on specific stimulus such as pups (Carlezon & Chartoff 2007). Future studies could incorporate stricter motivational testing procedures to better answer the question of whether cocaine-induced MB deficits are a function of reduced motivation to care for pups. If it is found that motivation to care for pups is truly lacking in CC-treated dams, new studies could attempt to prevent or circumvent changes in motivation in the postpartum.

7.2.2 Cocaine-Induced Affective Disorders in the Postpartum

Although confirmation of these results using other measures of anxiety-like behavior in rodents (i.e. elevated plus maze, light-dark box, social interaction testing) is needed, this initial report of inducing postpartum anxiety in rodents may provide a model to investigate neurobiological mechanisms of these behavioral changes. Replication of these results with other depressive-like behavior studies such as anhedonia (i.e. sucrose consumption or intracranial self-stimulation) or a classic learned helplessness (Porsolt) version of the swim test, could confirm that CC treatment reduces depressive like behavior in the postpartum. Given that CC treatment may change anxiety-like behavior differently across the postpartum period (see Appendix D, Figure 35), and cocaine exposure can impact a number of neurotransmitters that modulate anxiety-like behavior, such as serotonin and CRF (Corominas et al. 2010, Muller et al. 2007), these mechanisms deserve further exploration, especially as potential therapeutic targets for clinical populations.

Pilot studies have shown that on PND 1 and PND 2, many UN dams show a decrease in locomotor activity and increase in anxiety-like behavior (see Appendix D, Figure 35) compared to their own baseline behavior. This indicates that immediately postpartum (3-9 hours), dams are anxious when removed from pups. A great deal of variability was observed in this measure, which could be function of subtle differences in the timing of the experiment, time from

parturition, and/or endocrine signaling. Parturition causes a number of rapid physiological, endocrine and neurological changes to occur, many of which are reaching a new homeostasis within the first several hours after birth (Slattery & Neumann 2008, Weiss 2000). These changes could have large impact on the behavior of the animal, including anxiety and depressive-like behaviors (Zonana & Gorman 2005). Although we did not test endocrine levels following testing on PND 1 or PND 2, future studies could address this to determine what if any role they play in OFT behavior immediately postpartum.

Nevertheless, on PND 1, CC-treated dams show decreased anxiety-like behavior compared to their own baseline and compared to UN dams, this is intriguing as it is possible the CC-treated dams may have residual circulating cocaine at this time. Although cocaine treatment ended on GD 20, metabolism of cocaine is greatly slowed during the last week of pregnancy, because of decreased n-demethylase activity (Guarino et al. 1969). We did not directly test cocaine or cocaine metabolites in these dams however, although it would be useful when interpreting these results. However, other work in the lab indicates that pups maintain cocaine and cocaine metabolites in their urine for up to 5 days postnatally (McMurray 2011); thereby providing an opportunity for dams to ingest small amounts of cocaine in the first postpartum days.

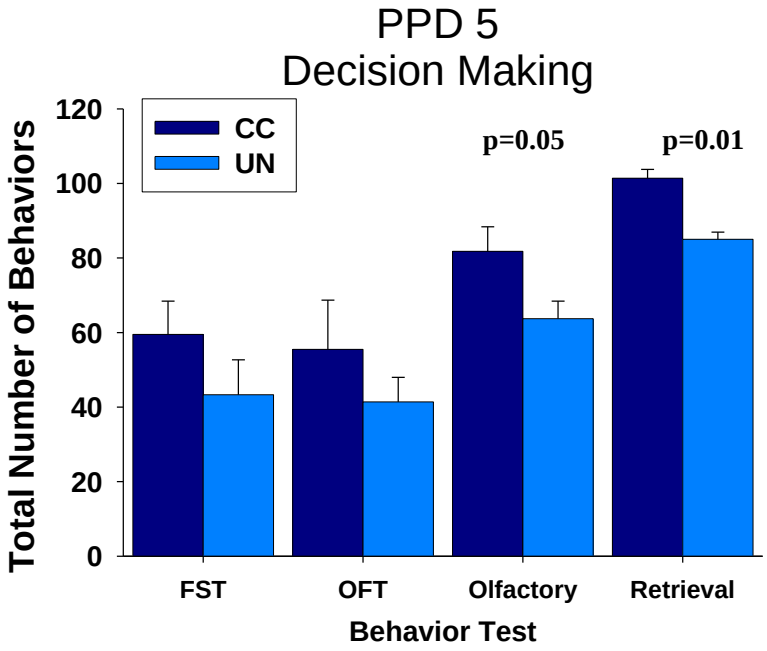

Figure 26. Total Frequency of Behaviors Across Tasks on PPD5.

Data presented as the group means ± SEM of total counted behaviors during a behavioral task. CC-treated dams show a greater number of behaviors exhibited across all tasks on PPD 5, differing significantly from UN dams in the olfactory ($p < 0.05$) and retrieval ($p < 0.01$). FST: forced swim test. OFT: Open Field Test.

7.2.3 Executive Function in Cocaine-Treated Mothers

The observed increased frequency of behaviors (increased switching between coded behaviors) across all the testing conditions (Figure 26) suggests a reproducible effect on decision making skills in CC-treated dams. This is a common problem among drug-abusers and can be exacerbated by stress (Orozco-Cabal et al. 2008). Cocaine-induced changes in prefrontal cortical (PFC) function may affect a mother's ability to appropriately choose and maintain pup-directed behaviors. The PFC's role in organizing behavior is critical for the transition to MB. As early as PPD 1, the cingulate cortex shows increased c-Fos expression in response to pups, and continues to respond to cues through the first week (Fleming & Korsmit 1996). Additionally, the infralimbic cortex responds to cues while the prelimbic cortex does not, suggesting the

importance of specific regionalization of circuitry. Pup suckling increases fMRI response in the medial and lateral PFC and insular cortex of lactating rats, an effect that is dependent on OT (Febo et al. 2005). EEG data suggest that mPFC activity changes in response to pup odors (Hernandez-Gonzalez et al. 2005). Pharmacological antagonism of sodium channels or activation of GABA in the mPFC has shown that this region is necessary for retrieval behavior of rat dams (Febo et al. 2010). These experiments did not change approach behavior towards pups, only the decision to retrieve them to the nest, indicating a change in motivation not investigatory behaviors. Excitotoxic lesion to the mPFC also disrupts pup retrieval, licking, and the overall pattern or order of MBs, indicating the importance of this region in working memory and attention in the postpartum period (Afonso et al. 2007). DA contributes to PFC function. DA levels are lower in rats in late pregnancy compared to virgin female rats (Olazabal et al. 2004), which may result in higher overall activity given that DA acts to inhibit activity in the mPFC (Peterson et al. 1990). Recently, high impulsivity has been tied to deficits in MB, which may be associated with alterations in mPFC function (Lovic et al. 2011). Since mPFC DA is an important mediator of impulsivity (Dalley et al. 2008) and can be disrupted by drug abuse, differences in behavioral organization during MB could occur following drug use (although this has yet to be directly tested).

Taken together, these studies offer some potential explanations for maternal-infant interactions following gestational cocaine treatment in rodents, however, they resulted in many more questions which are worthy of study.

APPENDIX A. CHAPTER 1: INTRODUCTION

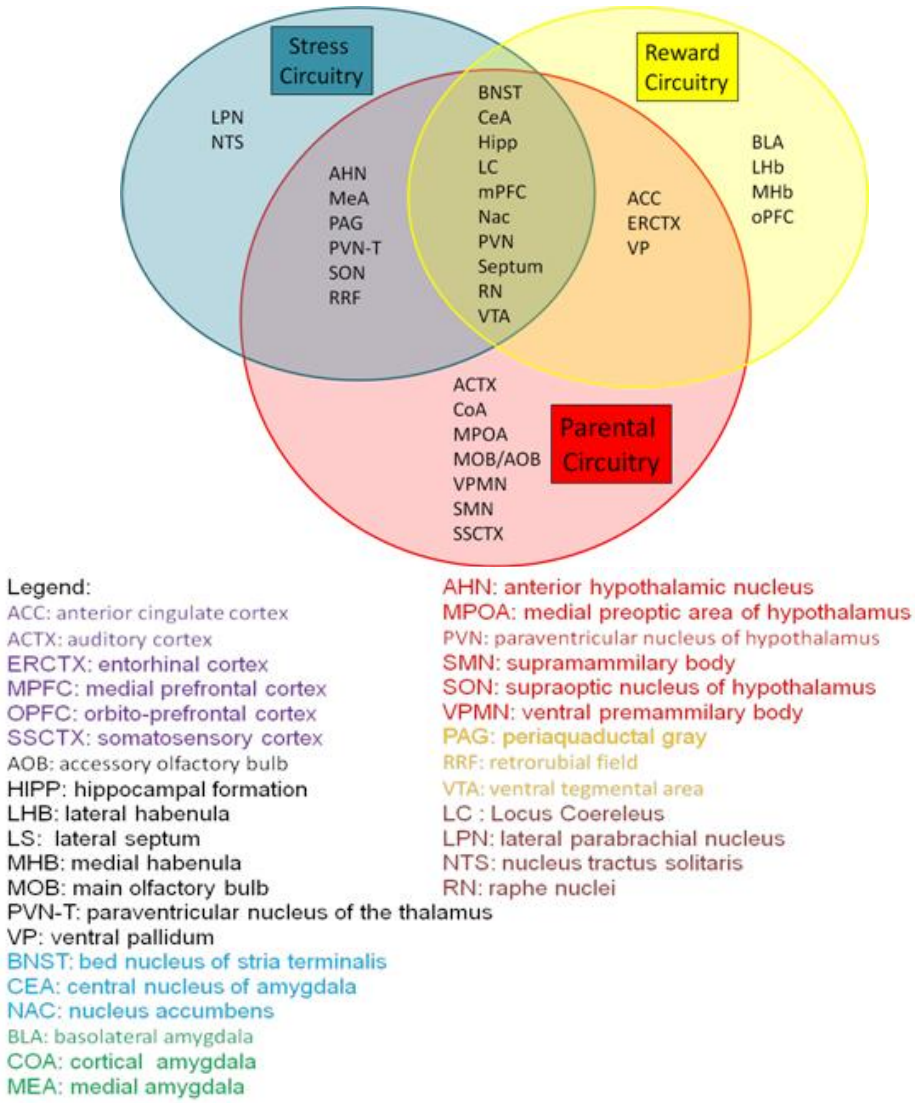

Figure 27. Maternal Circuitry Overlaps With Reward and Stress Circuitries.

Each circle encompasses brain regions which have been associated with specific cognitive roles. Parenting circuitry (green) shares many regions with stress (blue) and reward (yellow). The regions listed in the center have been implicated in all three circuits, suggesting that disruption in regions of circuit can have profound impact on the functioning of the connected circuits. Regions are listed in anterior-posterior anatomical order. Color coding in the legend indicates the anatomical brain systems each regions belongs to. Red: hypothalamus; Blue: extended amygdala; Purple: cortex; Green: amygdala; Orange: Midbrain; Brown: brainstem; Black: forebrain. Figure from Rutherford and Williams et al, 2011 (under revision).

APPENDIX B. CHAPTER 2: PROCEDURES AND METHODS

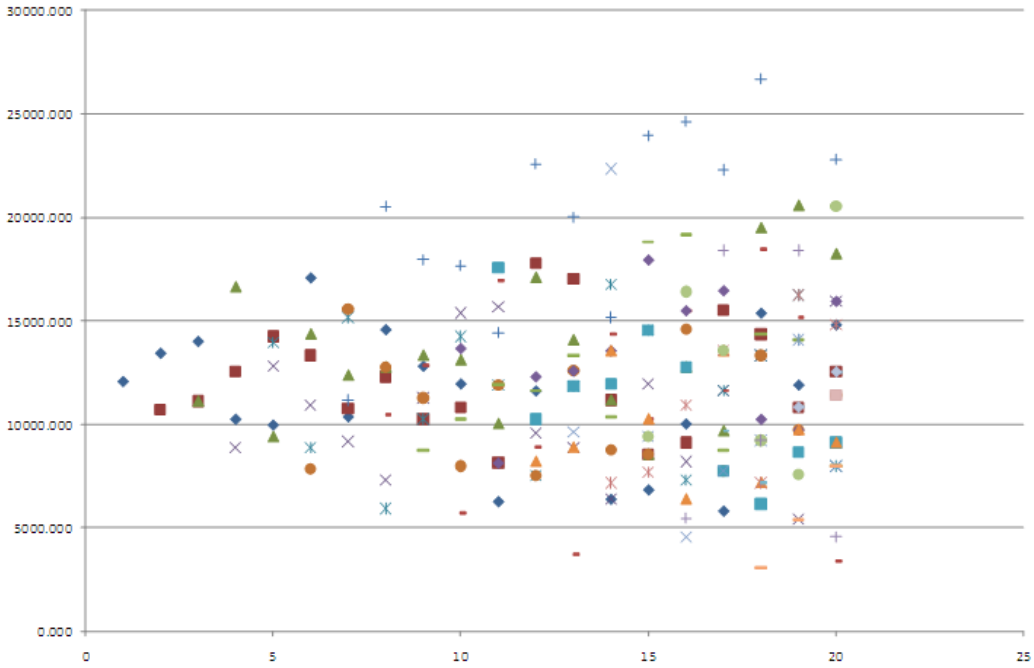

Figure 28. Cell Count Estimate Prediction Graph.

Each colored symbol represents the predicted estimates that would be obtained if objects of interest were counted every nth section. These data were based on physical counts of c-FOS stained nuclei in every other section of a single control animal using a set of determined parameters (50 um counting frame, 300 um2 grid size) to encompass the entire nucleus accumbens. Estimates are based on the simple random sampling used with the optical fractionators program within Stereoinvestigator software. This data indicated that counting every 7th section would allow counting the fewest sections while creating estimates with low variability. Therefore, we counted every 7th section throughout the entire NAc for this study.

Table 3 Brain Regions Dissected for OT Analysis.

Following a short thaw, brains were hand dissected into the labeled brain regions using the anatomical landmarks noted in the columns. Landmarks were chosen with the guidance of the Paxinos Rat Brain Atlas. (Paxinos & Watson 1997)

Region	Coronal Landmark Bregma Designation (mm)	Vertical Landmark	Horizontal Landmark
Olfactory bulbs Nucleus Accumbens (Nac)	2.70 to 0.48	Lateral to cingulum medial to external capsule	dorsal to corpus callosum
Anterior Hypothalamus	-.80 to -2.12	medial to lateral ventricles and lateral to optic chiasm	ventral to anterior commissure and 3rd ventricle
Amygdala (AMY)	-2.12 to -3.14	medial to lateral ventricles	ventral to rhinal fissure
Ventral Tegmental Area (VTA)	-5.20 to -6.04	lateral to accessory optic tract	ventral to rhinal fissure
Posterior Hippocampus (HIPP)	-4.16 to -6.04	lateral to optic tract	ventral to third ventricle

APPENDIX C. CHAPTER 3: RETRIEVAL PREFERENCE

Table 4. Home Cage Versus Test Cage Retrieval Latency.

The same animals were tested in both the test cage used for retrieval preference testing and their own home cage adapted for a 2 litter retrieval test. At least a 2 hour uninterrupted rest period separated the tests on each day. Dams were tested on multiple days (similar to test dam procedures). There was not a significant difference between the latencies in the home cage and the test cage.

Dam Treatment	Dam Number	Age	Pup	HOME CAGE	TEST CAGE
CC	160	1	CC	120	540
		1	CC	60	180
CC	163	1	CC	120	210
		1	CC	180	30
CC	160	3	CC	60	60
		3	CC	120	90
UN	276	3	UN	60	90
		3	UN	60	60
CC	163	3	CC	120	60
		3	CC	150	45
CC	154	3	UN	90	NA
		3	UN	120	NA
UN	267	3	UN	20	30
		3	UN	90	50
CC	163	5	CC	240	100
		5	CC	240	80
UN	276	5	UN	240	90
		5	UN	150	540
CC	160	5	CC	180	60
		5	CC	120	30
UN	265	5	UN	120	45
		5	UN	40	40
UN	267	5	UN	40	20
		5	UN	45	55
Averages				116.0416667	113.8636
T-Test p-value	0.47360467				

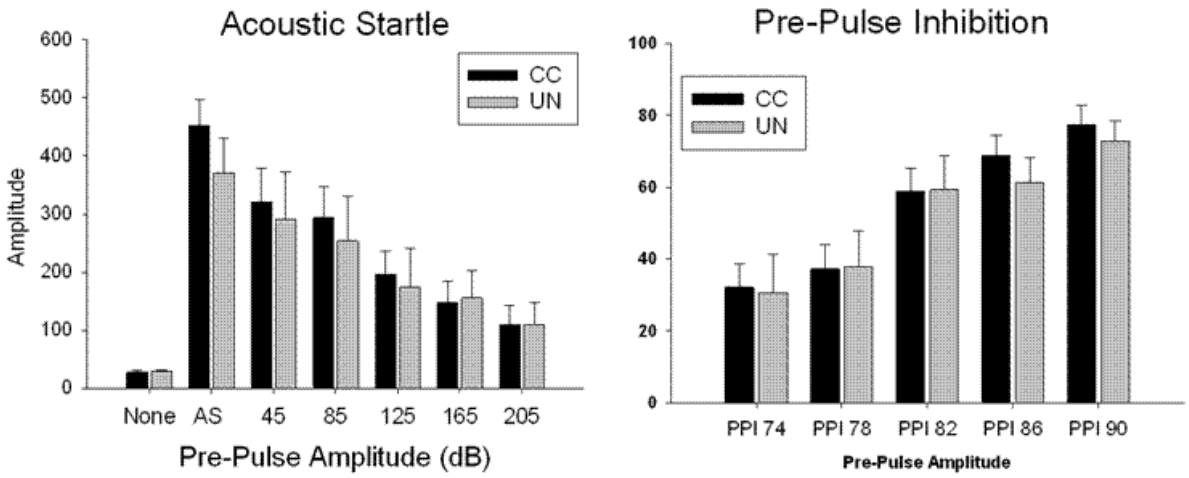

Figure 29. Acoustic startle response and Sensorimotor gating following gestational CC treatment.

Data presented as means ±SEM. There were no effects of CC treatment on startle response or percent pre-pulse inhibition response. These data indicate that CC dams do not have deficits in auditory sensation or sensorimotor gating. Rat dams were placed in a standard pre-pulse inhibition testing chamber on PPD5. Acoustic startle was measured as the amplitude of the transduced force exhibited by the rat immediately following the presentation of a loud sound. Detailed methods can be found in (Moy SS et al. 2006).

Table 5. Dam Olfactory Ability.

Olfactory ability was tested as described in Chapters 3 and 5. All test dams and PP dams were tested. Only a small percentage of dams did not retrieve at least one cereal piece during the GD 18 test. These dams all consumed cereal when returned to their home cages. These data indicate UN dams there are no differences between treatment groups in olfactory ability or gustatory response to sweet flavors.

Treatment Group	n	Percentage that did Not retrieve
CC	60	3.33
CS	18	5.56
UN	61	**11.47

Table 6. Retrieval Latencies.

Data presented as means± SD. Data presented is without regards to pup treatment condition. CS dams were faster to retrieve all 8 pups compared to UN and CC dams on PPD1 (*=p< 0.05). UN dams seemed faster to retrieve pups by PPD5, however these results were not statistically different.

	PPD	UN			CS			CC		
Latency to Retrieve First Pup	1	121.915	±	56.255	25.917	±	6.084	24.51	±	5.671
	3	60.432	±	41.86	43.367	±	10.753	108.845	±	61.039
	5	38.938	±	11.378	46.293	±	23.002	51.185	±	15.954
Latency to Retrieve Last Pup	1	319.93	±	87.238	136.325	±	14.618*	380.785	±	101.354
	3	231.623	±	83.269	269.41	±	70.201	290.1	±	79.238
	5	199.933	±	66.672	333.413	±	91.944	334.231	±	96.104

Table 7. Maternal Behaviors During Retrieval Test.

Data presented in means± SEM. There were several differences in Touching and non-pup directed behavior (Other). Asterisks indicate one treatment group is significantly different from the other two (*= $p < 0.05$).

		PPD 1		
Variable	Behavior	CC	CS	UN
Frequency	Other	19.667 ± 1.054	15.833 ± 0.705*	21.909 ± 1.224
	Crouch	0 ± 0	0.385 ± 0.14	0.364 ± 0.279
	Lick	5.9 ± 1.441	4 ± 0.892	4.818 ± 1.227
	Touch	35.9 ± 1.828*	30.385 ± 1.448	30.8 ± 1.937
	Group	6.8 ± 0.727	7.692 ± 0.414	6.273 ± 0.715
Duration	Other	301.15 ± 37.442	183.615 ± 21.416*	254.695 ± 26.138
	Crouch	0 ± 0	30 ± 13.332	5.5 ± 4.738
	Lick	89.37 ± 25.346	61.804 ± 19.325	73.16 ± 25.016
	Touch	429.935 ± 23.807	564.127 ± 24.799*	393.923 ± 56.753
	Group	2.765 ± 0.273	3.446 ± 0.261	2.827 ± 0.503
Latency	Other	0.585 ± 0.086	0.496 ± 0.048	0.523 ± 0.049
	Crouch	0 ± 0	805.542 ± 42.142	841.85 ± 54.042
	Lick	358.865 ± 41.65	373.292 ± 79.259	337.273 ± 75.514
	Touch	25.685 ± 5.635	28.481 ± 5.922	189.086 ± 87.895
	Group	66.66 ± 20.966	55.8 ± 7.384	233.95 ± 92.174
		PPD 3		
Variable	Behavior	CC	CS	UN
Frequency	Other	22.333 ± 1.421	23.333 ± 1.45	25.417 ± 1.104
	Crouch	0 ± 0	0.067 ± 0.067	0 ± 0
	Lick	3.75 ± 0.664	2 ± 0.498	2.846 ± 0.529
	Touch	29.091 ± 2.125	29.467 ± 1.191	35.167 ± 1.386*
	Group	5.583 ± 0.609	6.533 ± 0.584	7 ± 0.246
Duration	Other	424.042 ± 42.622	460.253 ± 29.892	431.483 ± 17.887
	Crouch	0 ± 0	0.627 ± 0.627	0 ± 0
	Lick	64.642 ± 16.589	33.13 ± 10.349	47.835 ± 13.314
	Touch	308.587 ± 46.485	340.183 ± 30.674	341.358 ± 35.088
	Group	1.817 ± 0.279	2.47 ± 0.441	1.919 ± 0.203
Latency	Other	0.375 ± 0.058	0.31 ± 0.018	0.412 ± 0.094
	Crouch	900 ± 0	899.373 ± 0.627	900 ± 0
	Lick	369.329 ± 89.287	413.617 ± 82.596	356.327 ± 83.721
	Touch	175.313 ± 86.147	44.073 ± 10.768	123.073 ± 73.694
	Group	206.837 ± 86.07	85.503 ± 22.171	153.323 ± 74.389

		PPD 5		
Variable	Behavior	CC	CS	UN
Frequency	Other	24.583 ± 1.96	22.933 ± 1.201	23 ± 0.944
	Crouch	0.25 ± 0.179	0.067 ± 0.067	0 ± 0
	Lick	3.083 ± 0.866	2.467 ± 0.389	2.636 ± 0.472
	Touch	31.273 ± 2.646	30.467 ± 1.46	32.333 ± 1.103
	Group	6.636 ± 0.472	6.4 ± 0.496	6.917 ± 0.529
Duration	Other	476.067 ± 41.322	449.063 ± 28.971	380.208 ± 29.073
	Crouch	23.133 ± 19.325	3.333 ± 3.333	0 ± 0
	Lick	51.196 ± 14.179	58.853 ± 11.871	55.392 ± 14.315
	Touch	260.571 ± 39.3	304.053 ± 30.187	419.067 ± 28.624*
	Group	2.404 ± 0.385	2.263 ± 0.19	2.513 ± 0.34
Latency	Other	0.354 ± 0.047	0.307 ± 0.029	0.375 ± 0.064
	Crouch	873.35 ± 22.79	896.517 ± 3.483	900 ± 0
	Lick	467.167 ± 106.19	445.673 ± 69.526	353.75 ± 79.733
	Touch	46.621 ± 16.747	46.99 ± 22.983	40.008 ± 11.351
	Group	59.445 ± 16.99	69.59 ± 26.291	55.633 ± 10.934

APPENDIX D. CHAPTER 4: ENDOCRINE CONTROL AND STRESS RESPONSE

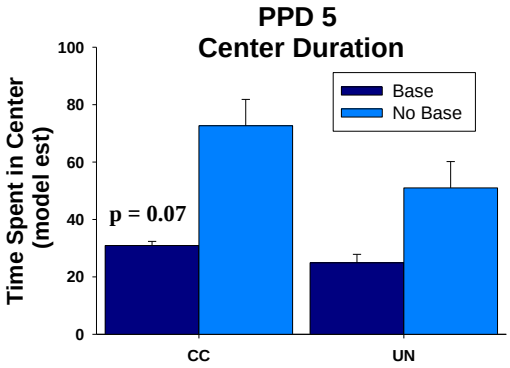

Figure 30. Effect of Habituation on Center Duration.

Type 1 and Type 2 dams (tested for baseline OFT behavior) were compared to a separate set of dams (first exposure to OFT chamber) for PPD5 OFT behavior. Data presented as means ± SD.

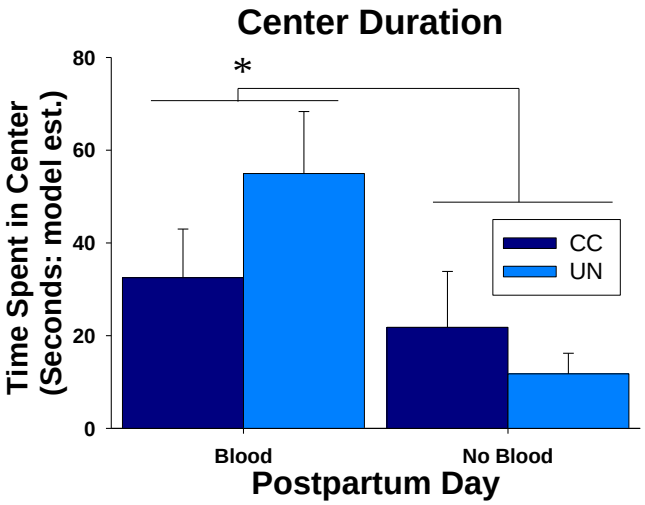

Figure 31. Effect of Blood Draw on OFT Behavior.

Data presented as means ± SEM. Type 1 dams (labeled Blood) had increased center duration compared to Type 2 dams (labeled No Blood) (*= $p < 0.05$). There was no interaction with treatment. Therefore data were pooled together for PPD 5 OFT analysis.

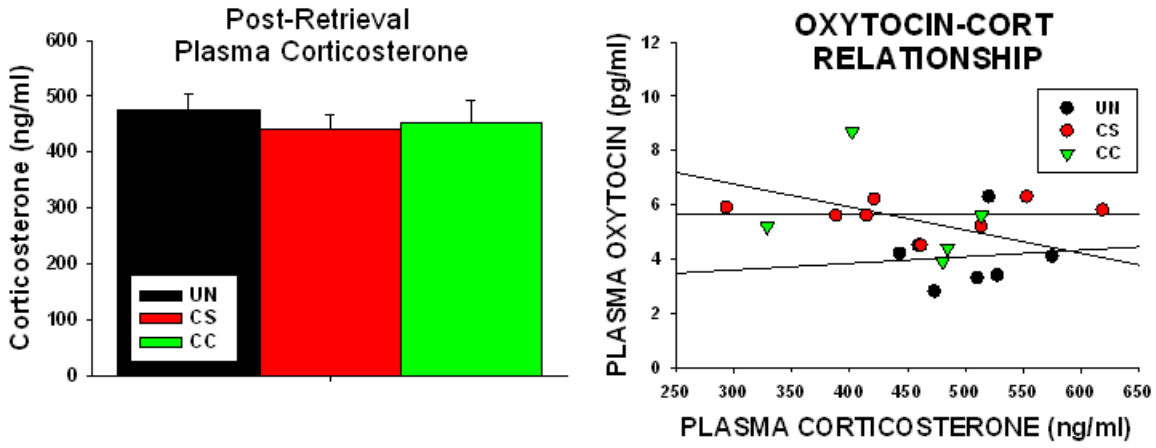

Figure 32. Endocrine Response to Retrieval Test.

CORT levels following retrieval were unaffected by CC or CS treatment. CORT levels were higher than baseline (compare with Figure 16 a in text). There was no relationship plasma CORT and OT levels in any treatment group.

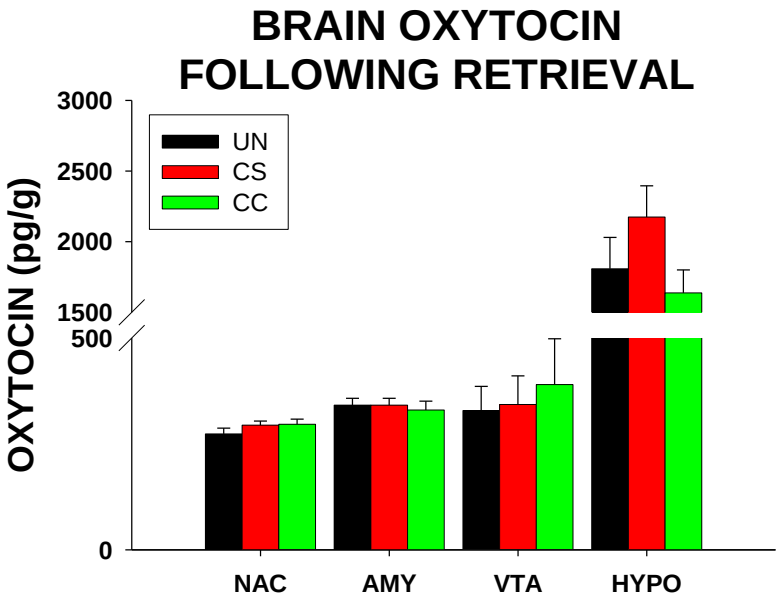

Figure 33. Brain OT following Retrieval Test.

Data presented as means ± SEM for each treatment group. No significant differences were observed.

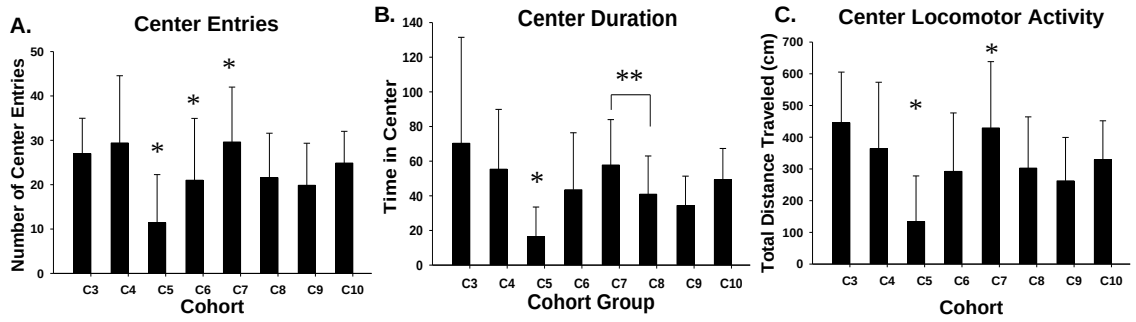

Figure 34 Baseline Open Field Testing.

A.) Number of times the rat entered the center. B.) Time spent in the center. C.) Locomotor distance traveled. *= significantly different from either adjacent cohort. **= significantly different from each other. C: Cohort group

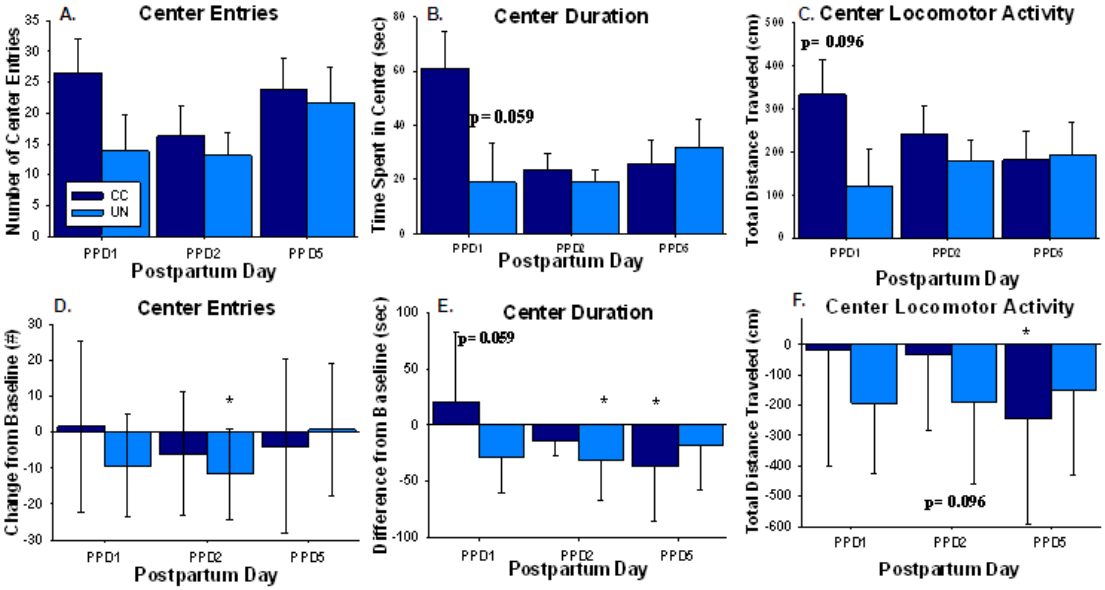

Figure 35. Postpartum Open Field Test Center Behavior.

A-C.) Graphs comparing results on each test day. Data presented as means ± SEM. D-F.) Change from Baseline. Data presented as means ± SD. *= $p < 0.0125$.

PPD1:

After adjusting for the differences in baseline measures, CC dams spend significantly less time in the wall compartment (p=0.03) and correspondingly a trend is observed for spending more time in the center (p=0.056). CC dams exhibit a trend for greater distance traveled in the center (p=0.09). When compared to their own baseline measures, CC dams show slower center velocity (p=0.0204), shorter wall duration (p=0.0046), less wall total distance traveled (p=0.0018) and slower wall velocity (p=0.0139). UN dams exhibit slower center velocity (p=0.0326) and a trend for less wall total distance moved (p=0.0833). CC dams reduced their activity more compared to their baseline measures than UN (p=0.0333). All dams were significantly slower in the Center [CC (p=0.0204); UN (p=0.0326)]. The wall velocity was lower than baseline for CC and UN groups, but the difference is significant for CC (p=0.0139). There were no differences in autonomic responses between CC or UN dams. There were no differences in autonomic responses between CC or UN dams.

PPD2:

There are no differences in open field behavior or autonomic responses on PND2 between UN and CC dams. When changes from baseline measures are considered, CC dams show less wall total distance (p=0.0119) and slower wall velocity (p=0.0273). UN dams have shorter center duration (p=0.0206) and longer wall duration (p=0.0209). UN dams show trends for fewer center entries (p=0.0599), wall entries (p=0.0518) and less center total distance moved (p=0.0901). CC dams showed greatly reduced distance traveled, a larger reduction than was observed in UN dams, however this difference did not reach a statistical significant level (p=0.0833

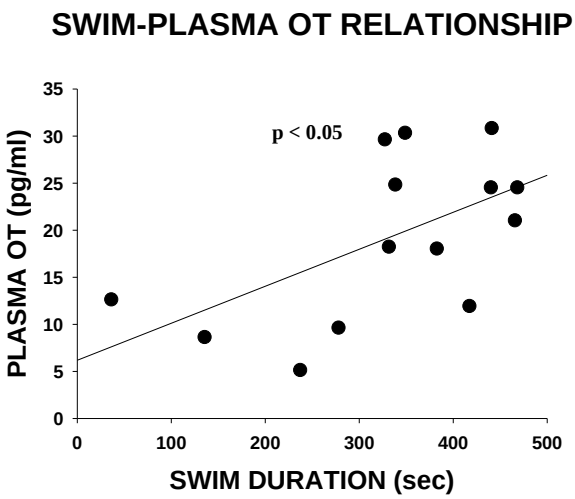

Figure 36. Relationship Between Swim Time and Plasma OT levels.

No treatment effects were observed so data is pooled across treatment groups for presentation. There was a significant positive relationship swim duration and plasma OT ($p < 0.05$).

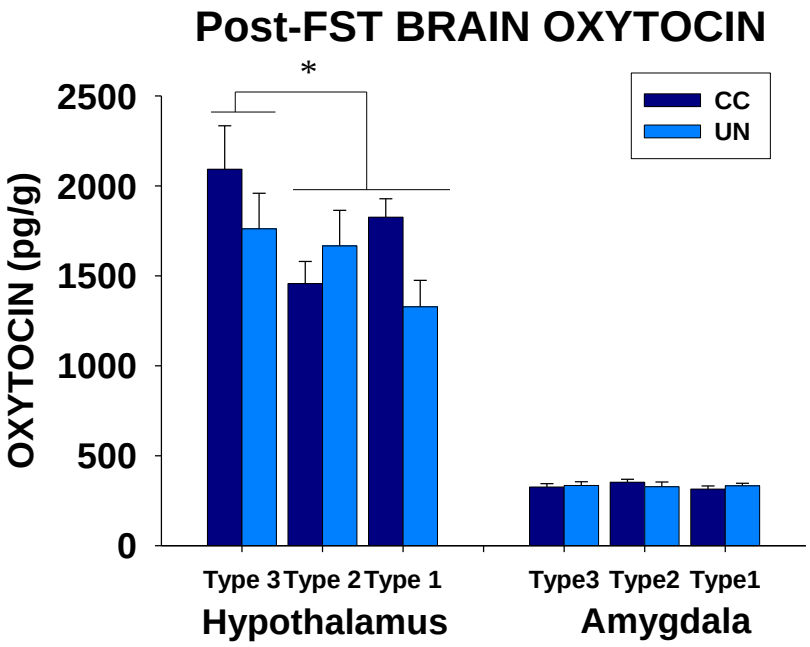

Figure 37. Brain levels of OT following FST

Data presented means ± SEM for each treatment group. Type 3 dams had significantly higher levels of OT in the hypothalamus compared to Type 1 and type 2 dams (*= $p < 0.05$).

APPENDIX E. CHAPTER 5: OLFACTORY PREFERENCE

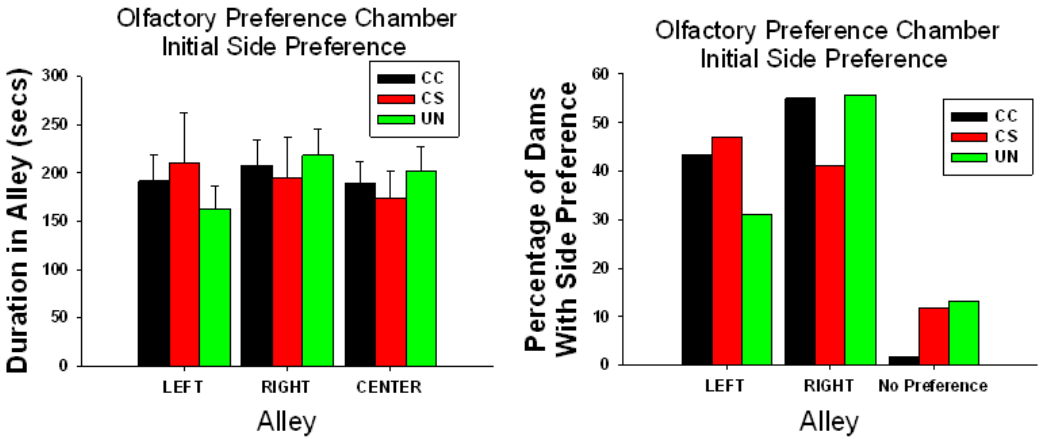

Figure 38. Gestational Day 19 Olfactory Chamber Side Preference.

On left, average duration spent in each alley. There were no main effects of alley or gestational treatment. On right, dams were categorized in to groups which preferred left or right side. A large number of females preferred one side or the other, however there was no overall preference by all dams for one side and no effect of treatment on this measure.

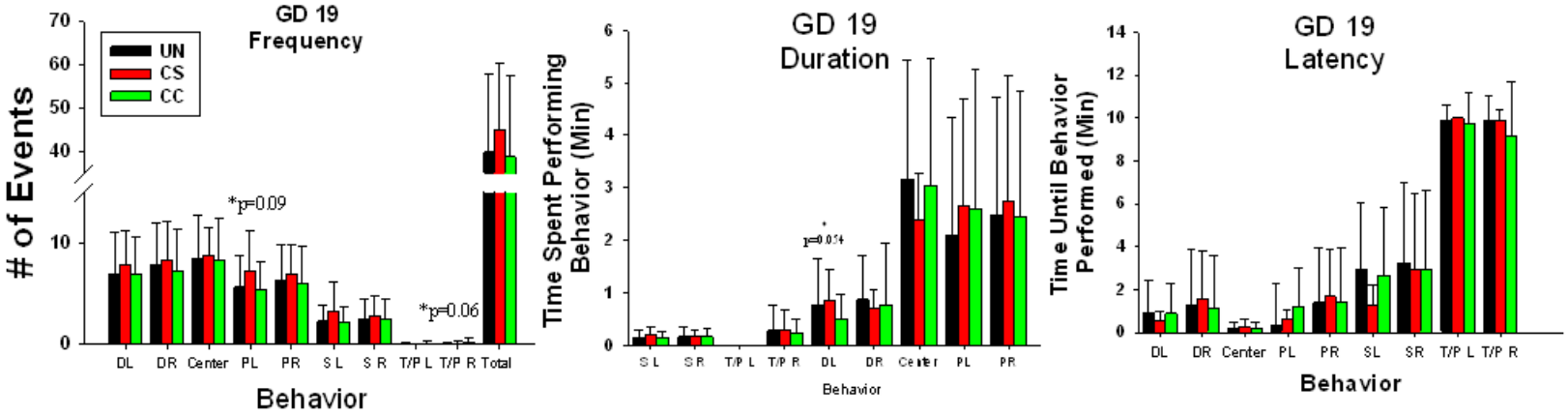

Figure 39. Specific behaviors of Olfactory Chamber Habituation Testing.

There were trends for small effects due to gestational treatment (indicated on graphs). DL: distal left. DR: distal right. PL: proximal left. PR proximal right. S L: sniff left. S R: sniff right. T/P L: touch/push left. T/P R: touch/push right. Total: the sum of all behaviors (interpreted as a measure of locomotor activity).

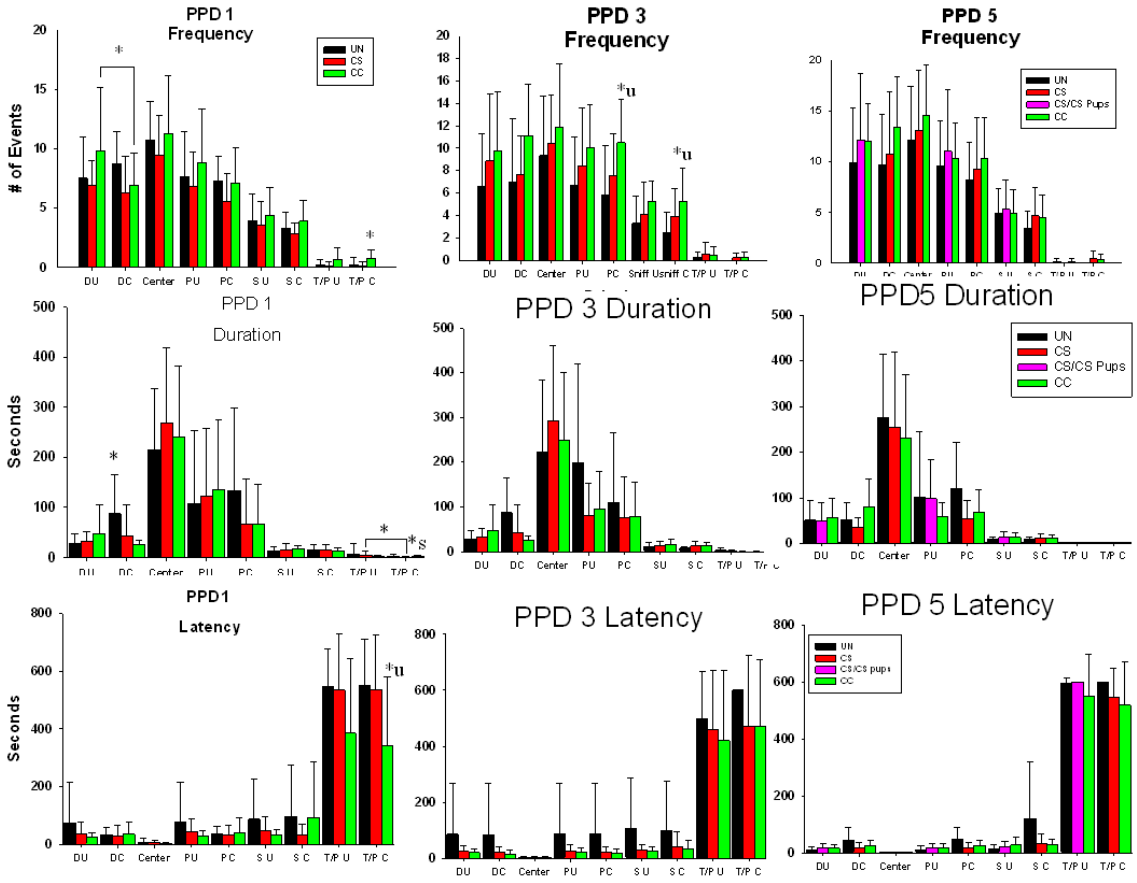

Figure 40. Specific Behaviors in Olfactory Preference Behavior Testing Across Test Days.

All data is presented as means ± SD on graphs on next page. Column on the left shows all PPD1 data. The center column shows PPD3data. The column on the right shows, PPD5 behavior. All is Frequency data across days as mean number of recorded events shown in the top row, Duration is shown in the middle row, Latency data is shown on the bottom row. For PPD 5 graphs, CS dam reaction to CS pup urine has been highlighted in pink, CS reaction to CC urine remains red. DU: distal UN alley. DC: distal CC alley. PU: proximal UN alley. PC: proximal CC alley. SU: sniff UN alley. SC: sniff CC alley. T/P U: touch/push UN alley. T/P C: touch/push CC alley. *u= different from UN dams $p < 0.05$. *s= different from CS dams $p < 0.05$. Lines indicate within dam differences in response to pup urine alleys. This was only observed on PPD 1.

PPD	Treatment	Habituation Time	Separation Time	Test Length
1	CC	0:48 ± 0:29	0:24 ± 0:06	0:12 ± 0:00
	CS	0:27 ± 0:06	0:22 ± 0:03	0:12 ± 0:00
	UN	0:33 ± 0:08	0:27 ± 0:06	0:12 ± 0:01
3	CC	0:29 ± 0:22	0:18 ± 0:03	0:12 ± 0:01
	CS	0:22 ± 0:08	0:18 ± 0:03	0:12 ± 0:00
	UN	0:26 ± 0:12	0:21 ± 0:05	0:12 ± 0:00
5	CC	0:32 ± 0:20	0:21 ± 0:05	0:12 ± 0:01
	CS	0:27 ± 0:08	0:21 ± 0:05	0:12 ± 0:00
	UN	0:43 ± 0:30	0:24 ± 0:08	0:12 ± 0:01

Table 8. Procedural Results for Olfactory Preference Testing.

There were no significant differences between treatment dams in the procedures. Data presented as mean ± SD rounded to minutes.

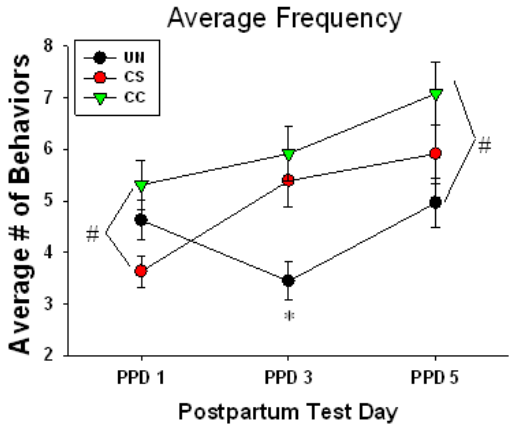

Figure 41. Average total frequency of behaviors.

Data presented as means ± SEM. CC dams had greater locomotor activity on PPD1 compared to CS dams and on PPD5 compared to UN dams (#= $p < 0.05$). UN dams showed less locomotor activity on PPD3 compared to CC and CS dams (*= $p < 0.05$).

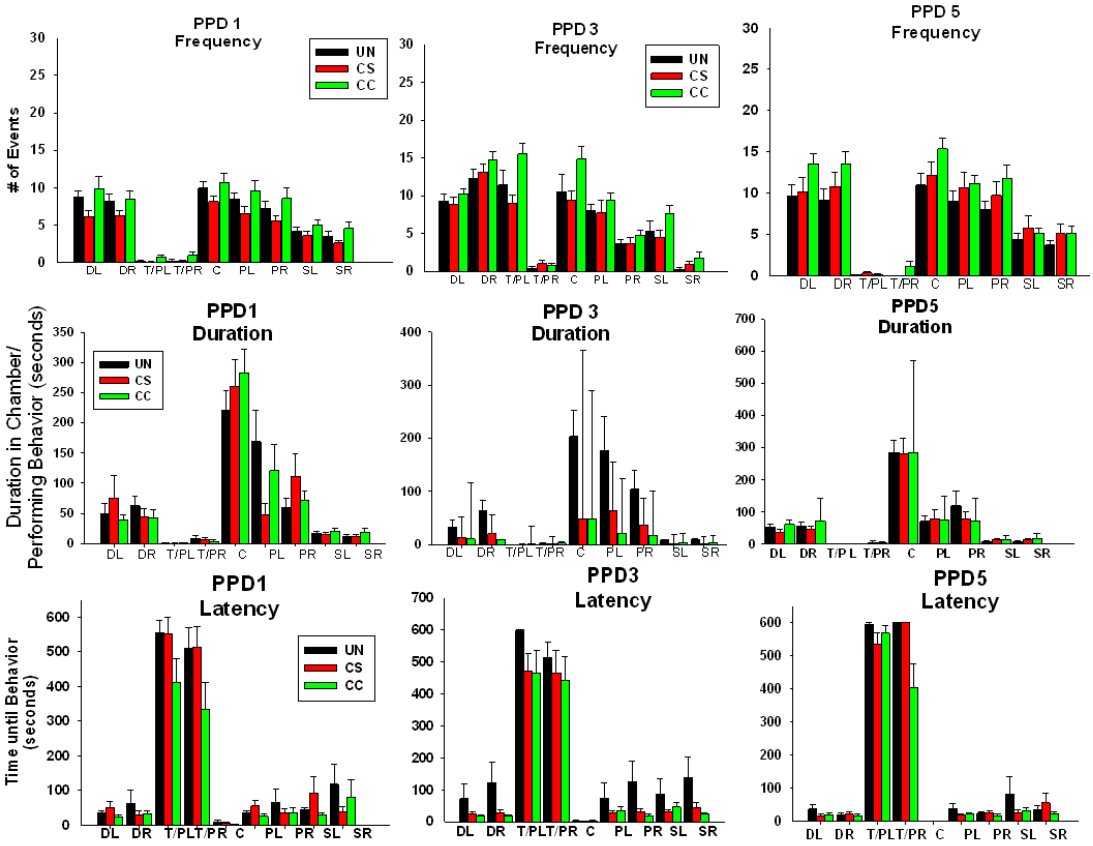

Figure 42. Specific Behaviors in SIDE Preference Behavior Testing Across Test Days .

All data is presented as means ± SD. Columns are represented by days. All is Frequency data across days as mean number of recorded events shown in the top row, Duration is shown in the middle row, Latency data is shown on the bottom row. For PPD 5 graphs, CS dam reaction to CS pup urine has been highlighted in pink, CS reaction to CC urine remains red. DL: distal LEFT alley. DR: distal RIGHT alley. PL: proximal LEFT alley. PR: proximal RIGHT alley. SL: sniff LEFT alley. SR: sniff RIGHT alley. T/P L: touch/push LEFT alley. T/P R: touch/push RIGHT alley.

Cell Counting of Nucleus Accumbens Shell

A separate analysis was performed to analyze the nucleus accumbens shell. Pictures of Nucleus accumbens sections were taken using a Nikon Eclipse E400 microscope at 10X magnification. The anterior commissure was visualized as the anatomical marker using Rat Brain atlas (Paxinos & Watson 1997). The shell was defined as the most medial portion of the image (see Figure 23). While the markers were not necessarily exact, the markers were consistently drawn based on distance from commisure. Darkened c-fos stained nuclei in the NAc shell were manually marked with the program Image J (Rasband 2011). These measurements of color density, along with measurements of background optical density, were used to determine if the marked cells were properly in focus by using a color density threshold. The threshold was set at 85% of background tissue values for each image section. Data were cleaned and averaged across sections (3-5 slices) for each animal.

A 2-way ANOVA revealed that CC pup urine compared to UN pup urine elicited a greater average number of c-FOS positive cells in the nucleus accumbens shell ($p < 0.05$).

We however observed that CC urine seems to be capable of eliciting a greater response in the nucleus accumbens shell compared to UN urine, which would suggest that chemical cues from the pups are recognized by dams, and thus potentially affecting MB towards CC pups. However, no observable behavioral preference or avoidance of CC pup urine cues was observed on PND5. Another possible explanation for this effect is that our

freezing procedure may have caused subtle differences in chemical make-up of urine (Hoffmann et al. 2009), and this could be different in CC pup urine.

Pilot studies investigating c-FOS staining in the VTA between

CC and UN dams exposed to UN urine were performed using Image J manual counting as well. No differences were observed average c-FOS cells.

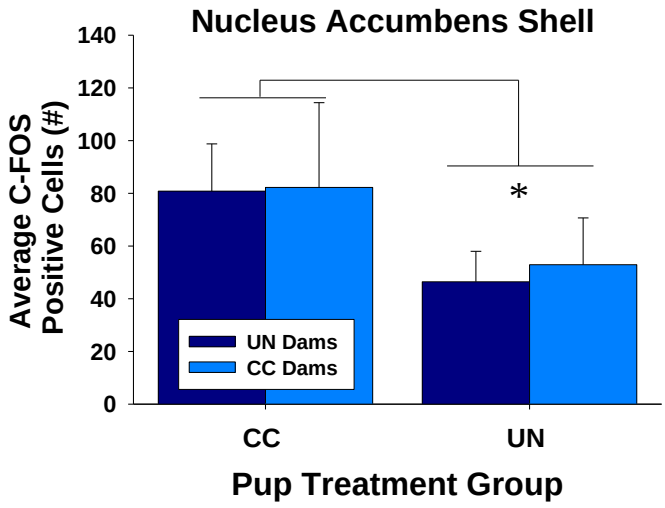

Figure 43. Average c-FOS count in Nucleus Accumbens Shell.

Data presented as means ± SEM. Cells were manually counted. CC pup urine elicited greater activation than UN urine ($p < 0.05$).

Treatment Group and Cotton Ball	Average number of c-fos stains per section per animal	Difference in c-fos stains from control
Untreated Dam given UN urine	24 ± 23	21 ± 23
Untreated Dam given No urine (control)	3 ± 4	
Cocaine Dam given UN urine	18 ± 19	5 ± 19
Cocaine Dam given No urine (control)	13 ± 12	

Table 9. VTA c-FOS Staining Following Exposure to UN urine.

N=4/group. UN represented in blue. CC dams represented in red.

APPENDIX F. CHAPTER 6: PUP CUES RELEVANT TO MATERNAL CARE

Pup Treatment	Age	Nesting Temp	Heat Pad Temp	Amount of Separation Time (Test Time -Time from Dam)	Amount of Cold Time (Test Time - Time From Heat Pad)	Start Temp	Temp Difference 1 (Start-Heat Pad)	Test End Temp	Temp Difference 2 (End - Start)	Band 1	Band 2
CC	1	30.61	31.99	34.86	12.86	26.77	-5.18	27.63	0.87	89.81	64.35
UN	1	29.74	31.54	35.59	12.67	26.73	-4.90	27.84	1.11	91.45	66.88
		**	**			**				**	**
CC	3	31.95	34.14	31.62	14	29.64	-4.47	29.30	-0.35	68.90	53.79
UN	3	31.54	33.95	32.03	13.26	29.66	-4.51	29.43	-0.21	65.07	47.54
CC	5	32.86	34.78	36.45	13.62	30.99	-3.74	31.10	0.15	76.14	55.93
CS	5	32.15	34.51	35.00	13.95	30	-5	30.56	1	64.11	46.94
UN	5	31.26	33.40	36.53	13	29	-4	29.71	1	75.03	52.62

Table 10. USV Collection Procedural and Temperature Data.

All temperature data is presented as degrees Celsius. Time data is presented as minutes. There were no differences in procedural times. There was no effect of treatment on temperature or temperature loss at any point. PND 1 pups had significantly lower body temperatures when taken from the nest, removed from the heat pad, and when the recording started. The total numbers of sounds recorded from PND 1 pups were higher than other age pups. (= p< 0.01, PND1 vs PND 3 and 5).**

Table	Prenatal Treatment											
	Test Dams								Pup-Provider Dams			
	CC			UN			CS		CC		UN	
Sample size	13			13			16		32 - 38		49 - 54	
GD 0	242.62	±	7.787	234.69	±	6.97	244.133	±	6.765	239.132	±	4.041	240.759	±	4.025
GD 20	370.15	±	8.715	373	±	7.404	388.8	±	8.481	367.342	±	5.142*	387.111	±	4.201
PPD 1	280.67	±	6.444	278.23	±	4.184	291	±	6.019	273.25	±	3.909*	292.216	±	3.46
PPD 3	282.23	±	5.305	284.25	±	3.668	294.333	±	7.281	280.139	±	3.248*	293.566	±	3.335
PPD 5	290.75	±	6.892	291.6	±	4.287	297.385	±	6.61	286.531	±	3.55*	301.583	±	3.241
1st Week Wt Gain	21.538	±	1.849	26.583	±	1.932	21.533	±	2.571	21.395	±	1.795*	26.667	±	1.09
2nd Week Wt Gain	38.231	±	1.545^{CS}	45	±	2.378	47.286	±	3.435	38.605	±	1.345*	46.093	±	1.02
3rd Week Wt Gain	67.769	±	3.74	71.538	±	3.345	77.071	±	3.828	68.211	±	2.281*	73.593	±	1.514
Total Pregnancy Wt Gain	127.54	±	5.35*	144.58	±	5.252	144.667	±	5.144	128.211	±	3.939*	146.352	±	2.391
Postpartum Wt Gain	11.545	±	2.06	10.333	±	2.991	9.615	±	2.294	14.969	±	1.952*	8.082	±	1.458

Table 11. Gestational Weight Gain Results.

Test dams and pup-provider dams are represented separately. Weights were collected every day of pregnancy. Asterisks indicate CC differs from UN and CS (p < 0.05). Superscripted CS indicates the CC dams differed CS dams only (p< 0.05).

	Prenatal Treatment		
	CC	UN	CS
Males	6.048 ± 2.048	6.909 ± 1.879	6.625 ± 1.857
Females	7.234 ± 2.228	7.164 ± 1.943	7.125 ± 1.586
Litter Wt	86.702 ± 13.998	86.388 ± 20.087	91.688 ± 11.265
Litter #	14.368 ± 2.421	14.033 ± 1.939	13.8 ± 1.612
P1 Culled Litter Wt	64.8 ± 9.34	67.333 ± 8.098	66.286 ± 3.474
P1 Pup	6.282 ± 0.466*	6.565 ± 0.425	6.6 ± 0.353
P3 Litter	75.2 ± 8.591	77.723 ± 9.022	79.688 ± 5.63
P3 Pup	7.446 ± 0.669	7.74 ± 0.804	7.773 ± 0.577
P5 Litter	99.231 ± 10.406	99.852 ± 10.065	102.929 ± 6.545
P5 Pup	9.961 ± 0.918	10.014 ± 0.904	10.277 ± 0.678
Weight Gain	4.543 ± 2.339	3.807 ± 2.478	3.2 ± 1.322

Table 12. Pup Weight Data.

All data presented as means ± SEM. The number of males, females and total number of pups were counted on P1. Regardless of number of pups, litters were culled to 10 pups. Pup weight was calculated by averaging litter weight/number of pups. P: postnatal day.

	PND 1		PND 3		PND 5		
	CC	UN	CC	UN	CC	CS	UN
Number of USVs							
Total	27 ± 2.732	28.82 ± 2.889	39.465 ± 5.795	56.429 ± 9.571	51 ± 5.002	54.83 ± 12.53	46.3 ± 6.333
MF	2.282 ± 0.435	2.591 ± 0.499	6.302 ± 1.112	6.976 ± 1.408	12.33 ± 1.187	9.417 ± 1.794	8.966 ± 1.09
H2	0 ± 0	0.045 ± 0.032	0.186 ± 0.083	0.31 ± 0.116	0.455 ± 0.115	0.667 ± 0.256	0.452 ± 0.13
H1	0.744 ± 0.359	0.818 ± 0.368	1.07 ± 0.295	1.024 ± 0.222	2.318 ± 0.32	2.5 ± 0.557	2.774 ± 0.395
L1H2	0.026 ± 0.026	0.136 ± 0.062	0.442 ± 0.243	1.095 ± 0.276#	0.523 ± 0.185	1.083 ± 0.583	0.968 ± 0.355
L2	5.487 ± 0.624	5.114 ± 0.639	5.721 ± 0.776	8.19 ± 1.308	11.39 ± 1.856	8.75 ± 2.219	11.484 ± 3.509
L1H1	0.872 ± 0.297	1 ± 0.322	5.256 ± 1.672	15.071 ± 4.799#	5.233 ± 1.427	5.818 ± 1.872	3.7 ± 1.367
L1	17.56 ± 1.866	19.07 ± 2.068	1.791 ± 0.486	1.929 ± 0.683	21.32 ± 3.037	22.42 ± 6.329	16.733 ± 2.33
Call Amplitude (dB)							
Call Amp-Ave	23.42 ± 0.354	24.3 ± 0.448	26.739 ± 0.536	27.825 ± 0.689	30.05 ± 0.787	30.69 ± 1.41	29.137 ± 0.603
Call Amp-Max	34.43 ± 1.26	35.96 ± 1.303	40.054 ± 1.331	42.219 ± 1.403	49.39 ± 1.414	49.81 ± 2.869	49.55 ± 1.713
Call Duration (ms)							
Call Dur-Ave	35.26 ± 1.506	36.13 ± 1.43	38.666 ± 2.196	47.853 ± 3.806*	43.19 ± 1.596	48.78 ± 3.692	43.413 ± 2.18
Call Dur-Max	83.08 ± 11.79	90 ± 14.7	97.143 ± 9.955	122.14 ± 16.21	125.5 ± 9.718	122.5 ± 14.04	114.19 ± 10.26
Call Dur-Total	800.8 ± 81.84	857.7 ± 86.69	1202.1 ± 174.1	1679.3 ± 285	1898 ± 257.4	1625 ± 372.3	1624.8 ± 307.3
Call Frequency							
Call Freq	31.86 ± 0.562	31.97 ± 0.511	34.726 ± 0.913	36.764 ± 0.981	39.85 ± 0.858	43.38 ± 1.476	41.366 ± 1.416
Call-Freq-max	51.47 ± 2.831	49.34 ± 2.106	63.917 ± 2.895	68.433 ± 2.969	75.76 ± 2.131	77.38 ± 3.637	82.361 ± 2.507
Bout Characteristics							
Number of Bouts	12.31 ± 1.002	12.32 ± 0.906	10 ± 0.872	9.31 ± 0.676	10.7 ± 0.426	10 ± 1.187	10.667 ± 0.609
Calls/Bout-Ave	1.768 ± 0.113	1.832 ± 0.114	2.419 ± 0.186	3.075 ± 0.346#	3.139 ± 0.254	2.764 ± 0.317	2.73 ± 0.273
Calls/Bout-Max	4.615 ± 0.524	4.665 ± 0.517	6.698 ± 0.844	8.946 ± 1.428	9.477 ± 1.24	8.667 ± 1.959	10 ± 1.766
#= .05 <p<.1	* = p<0.05						

Table 13. Acoustic Characteristics of Litter Produced Ultrasounds.

Data presented as means ± SEM for each treatment condition and age. On PND 3, UN pups had a significantly increased average duration (*= p < 0.05), and marginally more frequent calls with L1 H1 or L1H2 peaks (#= p< .1).

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