A RODENT MODEL OF COCAINE'S EFFECT ON THE MOTHER INFANT DYAD

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ABSTRACT

MATTHEW S. MCMURRAY: A Rodent Model Of Cocaine's Effect On The Mother Infant Dyad
(Under the direction of Josephine M. Johns)

Cocaine abuse by women is correlated with a high incidence of child neglect and abuse, and young children prenatally exposed to cocaine show early signs of neurobehavioral stress, including excessive and high-pitched crying, increased state lability, decreased responsiveness to caregivers, stress-related behavioral differences, and poor social development. Research on the effects of in utero cocaine exposure on early brain development and behaviors that elicit maternal care is relatively sparse. Using a rat model of cocaine-induced maternal neglect, the goals of this dissertation were to first examine the impact of cocaine on the interactions between rodent mothers and pups and to determine whether specific elements of pup behavior may be altered by prenatal cocaine exposure to influence these interactions. The first experiment described here examined whether the effects of cocaine-induced maternal neglect extend intergenerationally and if the rearing environment (neglectful or nurturing) can alter the effects of prenatal cocaine on offspring. Results from this study indicated that cocaine-exposed pups elicited reduced maternal care from their rearing mother, regardless of that mother's drug history. Since rodent mothers attend to the specific stimuli of pups, such as vocalizations, body temperature, and olfactory cues, the next study was completed to examine the impact of cocaine on the cues utilized by pups to elicit care. Results from these studies suggested that prenatal cocaine-exposure influences thermoregulation and vocalization in the early postnatal period, either directly or perhaps in combination with the indirect effects of prenatal stress and malnutrition. A third experiment was also conducted to examine a number of chemicals in pup urine that may contribute to the elicitation of maternal care. The only chemicals of interest that were

detectable in urine were cocaine and its major metabolites, found in samples through postnatal day 3, suggesting that cocaine may still be pharmacologically relevant into the postpartum period and may influence the taste and smell of pup urine, thus potentially influencing the maternal response. Together, this dissertation suggests that cocaine impacts both members of the mother-infant dyad to alter these important social interactions, and highlights numerous targets of prenatal cocaine on infant behavior for further study.

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LIST OF ABBREVIATIONS

GD	Gestation day
PPD	Postpartum day
PND	Postnatal day
HPA	Hypothalamic-pituitary-adrenal
CC	Chronic cocaine
CS	Chronic saline
UN	Untreated
FGD	First generation dam
ANOVA	Analysis of variance
BAT	Brown adipose tissue
T_A	Air temperature
T_{IS}	Interscapular temperature
T_B	Back temperature
USV	Ultrasonic vocalization
DP	Dodecyl propionate
LC/MS	Liquid chromatography / mass spectroscopy

CHAPTER I

GENERAL INTRODUCTION

A Brief History of Prenatal Drug Exposure

Fetal exposure to drugs of abuse is arguably one of the more preventable causes of learning and developmental disorders, and exceeds Down's Syndrome and Autism in prevalence (Fombonne 2009). Fetal Alcohol Spectrum Disorder alone effects approximately 10 out of every 1000 births (May et al. 2001), a statistic that increases when considering exposure to other drugs of abuse. Even more startling is that despite a concerted public health effort, the number of infants born with in utero exposure to drugs of abuse (including alcohol and nicotine) is climbing (Office of Applied Studies 2008), as is the human and financial cost associated with such exposure. Recent estimates (Stade et al. 2009) of the cost of Fetal Alcohol Spectrum Disorder alone are as much as $11 billion (adjusted for 2002), or nearly half of the annual budget for the state of North Carolina. Clearly, prenatal exposure to drugs of abuse continues to pose an enormous public health and financial problem, and thus an important field for scientific study.

While a large body of research has been accumulating with respect to fetal alcohol exposure, less is known about exposure to other drugs of abuse, particularly stimulants, with cocaine being one of the most prominent. During the height of the cocaine epidemic of the 1980's and 90's an abnormally large number of infants were born to cocaine-abusing, primarily black, mothers. The consensus at that time was that these babies were hopeless cases. The public's already prejudiced opinion of the mothers of these infants spread to the infants themselves. This early opinion was fueled by statements from a number of prominent outspoken individuals, such as Boston University

President John Silber's statement lamenting the usage of tax dollars to care for "crack babies who won't ever achieve the intellectual development to have consciousness of God." Pulitzer Prize winning columnist Charles Krauthammer declared that cocaine-exposed babies would have "a life of certain suffering, of probable deviance, of permanent inferiority."

Clinical science finally began to address the issue of prenatal cocaine exposure to some extent in the 1980's to 1990's. Clinical research on the topic was not without its weaknesses, however. Physicians published case-reports on infants in the worst possible situations, born exposed to a number of drugs, to single mothers with a low socio-economic status, and having numerous other health issues. In 1985, one of the first scientifically designed studies was published indicating that infants of 23 cocaine users were more irritable and less interactive or engaged than non-exposed babies (Chasnoff *et al.* 1985). Even though several important controls were not employed (socioeconomic status, concurrent non-cocaine drug use, prenatal care, etc), many individuals considered these to be the definitive findings concerning these infants.

The National Institutes of Health had not funded large-scale clinical research to this point, largely because of the expense required for such projects. Journals were also more likely to publish papers that demonstrated an effect of prenatal cocaine than those showing no effect regardless of control issues in the late 1980s (Koren *et al.* 1989). A turning point came in a 1992 paper that stated, "predictions of an adverse developmental outcome for these children are being made despite a lack of supportive scientific evidence. Whatever the true outcome, we are concerned that premature conclusions about the severity and universality of cocaine effects are in themselves potentially harmful to children" (Mayes *et al.* 1992). Clearly, clinical science was just becoming aware of the need for well-designed research in this area.

Epidemiology

Although the epidemic of the 1980's may have come to a not-so-abrupt close, maternal cocaine usage has certainly not ceased, and remains a large public health concern. Cocaine has been,

and continues to be, one of this country's top illicit drug problems. The perceived availability of cocaine has remained relatively constant since 2002. In 2008, an estimated 12% of the American population, roughly 37,000,000 individuals, reporting use during their lifetime (Office of Applied Studies 2007; Office of Applied Studies 2008). This number is up from approximately 11% in 2007. Approximately 16 million women report use during their lifetime, and 1.9 million reported using during the past month. While these statistics may show a trend towards increasing usage, there have been some improvements, especially with regard to use by women of childbearing age (15-44 years old). In 2006, roughly 700,000 women of child-rearing age reported usage in the past month, while in 2008, this number dropped to a little over 500,000. The improvement is even more positive within pregnant women. The number of pregnant women using cocaine decreased from a reported 45,000 in 1992 to approximately 9,000 in 2008. While there have been some positive trends in cocaine use statistics, it is clear that cocaine remains an important public health and financial concern, especially for maternal and child health.

Effects of Prenatal Cocaine Exposure

Generally it is thought that there are two primary causes of the pathophysiological effects from prenatal cocaine exposure: the direct effects of cocaine on neurodevelopment, and its indirect effects resulting from vasoconstriction. Cocaine readily crosses the placental barrier, thus entering fetal circulation and directly affecting the central and peripheral nervous systems. Cocaine's mechanism of action in fetal brain is identical to its mechanism in adult brain, where it primarily blocks presynaptic monoamine uptake, effectively increasing extracellular levels of the neurotransmitters dopamine, serotonin, and norepinephrine. However, in contrast to the adult brain where cocaine alters signaling parameters in an already developed brain, fetal cocaine exposure is more likely to result in malformations of the developing brain, potentially disrupting the development of uncountable behavioral systems and biochemical pathways. These teratogenic effects depend greatly upon the time point of exposure, as well as the duration and dose. As new physiological

systems become functional, these developing systems are more susceptible to insult caused by teratogens, such as cocaine. Such vulnerable periods in development are summarized in Figure 1 below (adapted from (Selevan *et al.* 2000)).

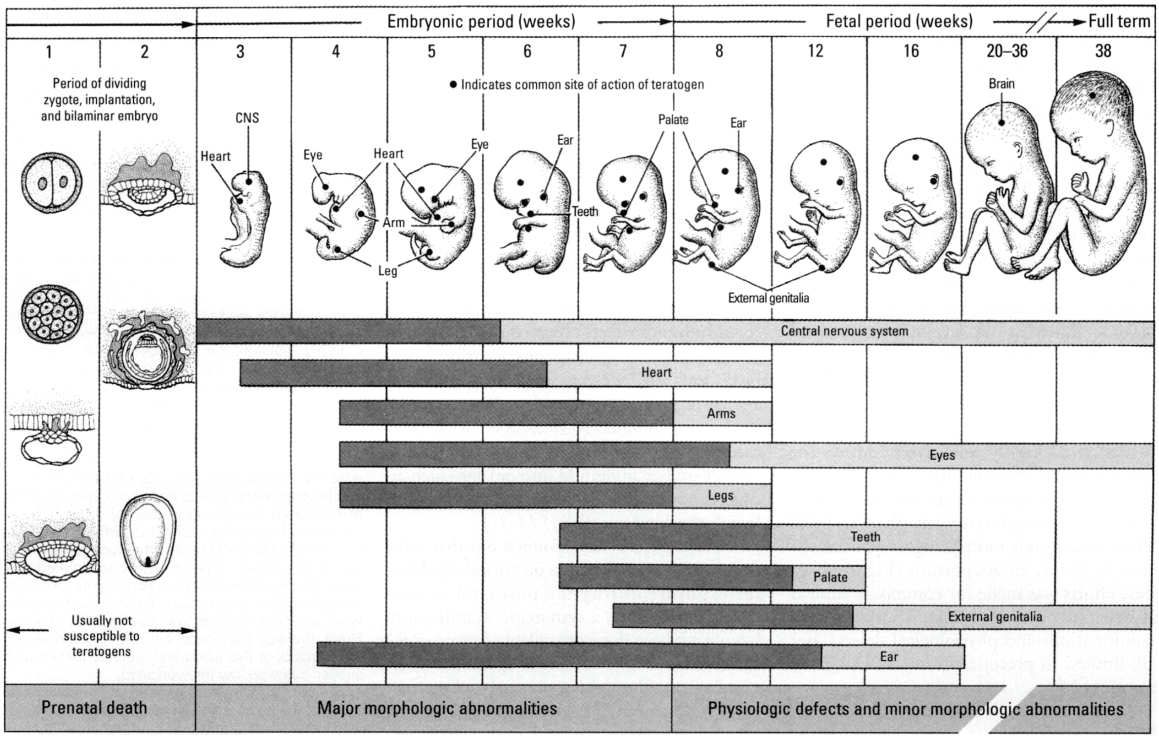

Figure 1.1. Schematic illustration of the vulnerable periods in human development. Dark gray denotes highly sensitive periods; light gray indicates stages that are less sensitive to teratogens.

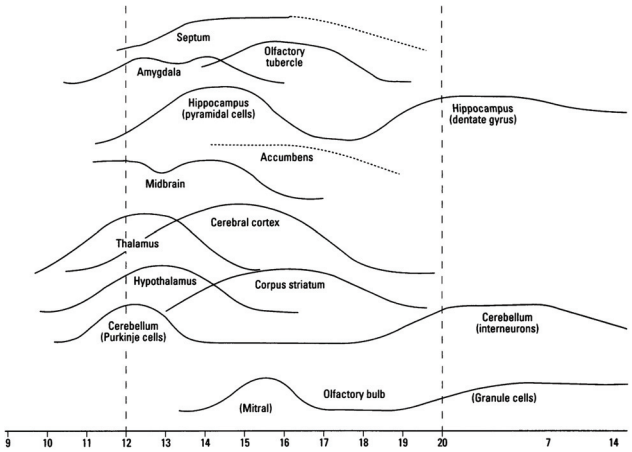

Figure 1.2. Critical periods for neuronal development in the neonatal rodent brain. Times are based on autoradiographic studies of specific structures over time. The vertical line at day 12 represents the approximate end of critical period for gross defects and the vertical line at day 20 indicates birth.

Especially vulnerable developmental periods exist not only in the whole organism, but within the developing brain as well. During central nervous system development, brain regions form non-synchronously, causing especially vulnerable periods of development for specific brain regions. Such periods in the rat are shown in

Figure 2 (Selevan *et al.* 2000). While each region may emerge anatomically at different developmental time points, their development tends to overlap to some degree. These periods of overlap include especially sensitive periods where exposure to teratogens can affect the development of a large number of brain regions, potentially resulting in widespread effects on postnatal behavior and health (highlighted in Figure 2, noted between the vertical dotted lines). In addition to such critical periods in brain formation, the early stages of brain development are also especially vulnerable to effects of environmental contaminants, as disruptions in neurogenesis and cell migration can have long lasting and often dramatic effects on the outcome of the developing brain, as seen in fetal alcohol exposure (Sulik 2005).

As discussed above, cocaine's primary mechanism of action results in reuptake inhibition and, thus initially increased levels of the neurotransmitters serotonin, dopamine, and norepinephrine, which in turn allow for both direct and indirect effects on development. Monoamines are among the earliest signaling molecules to emerge in development (Levitt et al. 1982; Puelles et al. 1998), and thus provide an important regulatory role as demonstrated in mouse knock out models of tyrosine-hydroxylase and dopamine-β-hydroxylase, the precursors to dopamine and norepinephrine, respectively. Knock-outs of these genes result in an almost complete loss of fetal viability (Kobayashi et al. 1995; Thomas et al. 1995; Thomas et al. 1998; Zhou et al. 1995). Dopamine plays a particularly important regulatory role in both neurogenesis and neuron migration to cortical regions (Bhide 2009). Additionally, serotonin works developmentally to influence the genesis, migration, and targeting of growing serotonergic neurons, which in turn can alter the development of other neuron types and even target tissues (Whitaker-Azmitia *et al.* 1996). Given the importance of these neurotransmitter systems in development, it appears likely that the developmental effects of cocaine work directly through these mechanisms.

Among the most robust physical findings in cocaine-exposed human infants is a reduction in head circumference compared to control infants (Bada *et al.* 2002; Chasnoff *et al.* 1998; Mirochnick *et al.* 1995; Zuckerman *et al.* 1989), potentially a direct physiological consequence of the impact of

cocaine exposure on brain development. These findings have been further explored in preclinical models using full-term cocaine exposure, which have demonstrated alterations in the development of both the neocortex (Akbari et al. 1994; Jones et al. 1996; Kosofsky et al. 1994; Lidow et al. 2001; Ren et al. 2004) and hippocampus (Baraban *et al.* 1999), as well as disruptions in central myelination (Wiggins et al. 1990). Prenatal cocaine exposure has also been shown to alter developmental neurogenesis, new neuron proliferation, and connectivity (Garg *et al.* 1993; Nassogne *et al.* 1995; Nassogne *et al.* 1998), effectively disrupting neuronal migration and thus cortical structure.

Secondary to its direct effect on monoamine levels, cocaine may work indirectly to alter development through its vasoconstrictive properties. An increased plasma concentration of catecholamines in the cocaine-using mother reduces placental blood flow, thus reducing fetal oxygen and nutrient supply and potentially causing hypoxia in the developing fetus. Catecholamine levels are also elevated in the developing fetus, resulting in fetal vasoconstriction and potentially resulting in further hypoxia in the developing brain. When coupled with the anorectic effects of cocaine, a reduction in fetal blood supply can have disastrous effects on development. In general, maternal cocaine usage leads to an increased likelihood of fetal resorption, and in those infants that survive, there is an increased likelihood that the infant will be Small for Gestational Age, born prematurely, and have reduced birth weight. These are likely the effects of in utero malnourishment. In addition to malnourishment, hypoxia activates the sympathoadrenal system through increases in pH, resulting in fetal stress.

Fetal distress may be a third emerging cause of cocaine's pathophysiological effects. While there is little empirical evidence to support this hypothesis, it is generally thought that elevated levels of fetal stress hormones following a stressor such as prenatal cocaine exposure can act upon the hypothalamic-pituitary-adrenal (HPA) axis to alter the neuroendocrine environment, potentially changing the set-point for endocrine-related behavioral phenotypes (Matthews 2001). As Lester and Padbury stated, "There are few settings in which gene-environment interactions are more profound, critical windows are of a narrower duration, and the latency to onset of effect is shorter, than the

influence of an adverse intrauterine environment on neuroendocrine and neurobehavioral functioning in the newborn" (Lester et al. 2009).

Researchers have recently turned to animal models in an effort to isolate the specific effects of prenatal cocaine from the various confounds associated with research in human populations (socioeconomic status, subject compliance, etc). Rodent research has revealed that rat pups of different ages that are prenatally exposed to cocaine differ in their endocrine response to tactile stimuli, stress responsivity, ability to elicit play solicitations from a normal conspecific (Wood *et al.* 1994; Wood *et al.* 1995), and exhibit abnormal social/aggressive behavior (Johns *et al.* 1994a; Johns *et al.* 1994b; Johns *et al.* 1999; Overstreet *et al.* 2000). Prenatal cocaine exposure has also been speculated to result in an altered ability to recognize social cues or relevant behavioral displays (Johns et al. 1995), as evidenced by a tendency towards asocial behavior and aggression. Many of these effects of prenatal cocaine found in rodent populations are similar to the effects of fetal stress, suggesting the validity of this theory. Fetal stress alone is associated with a number of developmental effects in rodents, including an elevated degree of "emotionality," deficits in play behavior, increased vocalizations, and impairments in discrimination, reversal learning, and memory. Additionally, human infants with fetal stress are often reported to be more irritable, anxious, and difficult to control. These last effects are particularly important as they relate to maternal-infant interactions. It is unknown if such effects of cocaine also exist in rodents, but should these effects translate between the two species it would suggest yet another potential phenotype targeted by prenatal cocaine.

Effects of Cocaine on the Mother-Infant Dyad

Development in most species involves at least two parties: the offspring and the parent. Our own anecdotal evidence suggests that even following an optimal uterine environment, there is still tremendous room for developmental disruption in the postpartum period due to poor maternal care. While subtle aspects of maternal care can affect development, perhaps the most dramatic example of how maternal care can influence development is in the extreme case of child maltreatment or neglect,

when maternal care is poorest. The effects of maltreatment are not only immediate, with maltreated children showing significant cognitive delay and weight gain compared to controls (Scarborough *et al.* 2009), but long term behavioral effects can also occur, often manifesting in adolescence or adulthood (Johnson *et al.* 1999).

Cocaine-use by human mothers during pregnancy is associated with a greater incidence of child neglect (Kelley 1992), deficits in mother/infant "bonding" (Burns *et al.* 1991), child abuse (Murphy *et al.* 1991), and placement in foster homes (Leventhal *et al.* 1997; Nair *et al.* 1997). Cocaine-using mothers have been shown to respond less to their infants (Mayes *et al.* 1997), and are more likely to show hostility during feeding or play interactions (Goldman-Fraser 1997; Light *et al.* 2000). However, clinical studies must always account for numerous confounds such as poor subject compliance, biases in sampling, and multi-drug exposure, thus limiting the interpretability of these findings. Animal models can more precisely target the impact of cocaine on maternal behavior without the many confounding variables inherent in human studies. A variety of cocaine treatment models have been explored in pregnant or postpartum rats, which include chronic (throughout the 21 day pregnancy), acute (single dose after delivery, 30 minutes prior to testing), or intermittent (two days every five days during pregnancy and lactation) at doses generally between 15-30 mg/kg. These studies observed an increased latency and decreased duration of nursing behavior in chronically, acutely (when cocaine is in their bloodstream) and intermittently treated groups, along with other general disruptions in maternal behavior (Elliott *et al.* 2001; Johns *et al.* 1994c; Johns *et al.* 1997b; Johns *et al.* 1998b; Nelson *et al.* 1998). The results from studies such as these suggest that all regimens of cocaine treatment seem to affect similar systems, though the extent of effect seems determined by the administration regimen (Johns *et al.* 2005a). Importantly, these effects are not primarily attributable to cocaine withdrawal (Johns *et al.* 1997b) or cocaine-induced hyperactivity (Johns *et al.* 1994c; Kinsley *et al.* 1994; Vernotica *et al.* 1996a; Vernotica *et al.* 1999). Significant effects begin to appear at a moderately high dose (30 mg/kg/day) of cocaine (Nelson *et al.* 1998), translating to approximately 1 gram of cocaine in a 150 lb woman.

Since many cocaine-exposed children experience adverse maternal care environments, as clinical data indicate, any behavioral/biological disorders caused by prenatal cocaine exposure may be exacerbated in these children (Eiden et al. 1999; Smith 1992; Zuckerman et al. 1993). Thus, there is likely an important interaction between any biological vulnerabilities due to the direct effects of prenatal cocaine exposure and any indirect effects of the postnatal environment. In other words, infants with prenatal cocaine exposure may be less capable of eliciting the optimal care from their mothers. Indeed, infants with prenatal cocaine exposure show poor state regulation (Eiden et al. 2009a; Schuetze et al. 2007; Schuetze et al. 2009b; Schuetze et al. 2009a), exhibit altered physiological responses to stimulation (Eiden et al. 2009a; Eiden et al. 2009b), are more excitable (Eiden et al. 2009a), and are generally less physiologically stable (Bendersky et al. 1998a; Bendersky et al. 1998b; Brown et al. 1998; Chasnoff et al. 1989; Chiriboga et al. 1993; Delaney-Black et al. 1996; Gingras et al. 1995; Jacobson et al. 1996; Karmel et al. 1996; Lester et al. 1998; Mayes et al. 1995; Mayes et al. 1996; Neuspiel 1995; Nulman et al. 1994; Regalado et al. 1996; Regalado et al. 1995; Sheinkopf et al. 2006b; Tronick et al. 1996). Given cocaine's likely effects on early brain development resulting from both prenatal exposure, as well as the secondary effects of altered maternal care, it is reasonable to suggest that individuals with prenatal exposure to cocaine may exhibit a different pattern of effects as adolescents and adults than they did as infants.

The Specific Aims of this dissertation examine how prenatal cocaine exposure affects stimuli related to early mother-infant interactions. Specifically, to determine: **1.** If dam cocaine treatment or pup prenatal cocaine exposure alters dam-pup interactions; **2.** If prenatal cocaine exposure alters pup-produced stimuli that could subsequently affect maternal behavior towards those pups; and **3.** How long cocaine and its metabolites are present in pup urine following parturition and if levels of additional urine chemicals are altered that may influence the odor or taste of urine. These experiments will be the first systematic evaluation of these issues. The long-term objectives are to determine how cocaine affects development in prenatally-exposed offspring, nurturing behavior in mothers, and subsequent mother-offspring interactions. While it is beyond the scope of this single proposal to

manipulate and determine differential response of mothers to various changes in pup-produced stimuli, future experiments will make these determinations. It is reasonable to suggest that drug-exposed infants seeking care from a non-nurturing caregiver may have difficulty responding to their mother/caregiver, as well as forming normal social bonds, which if true in clinical and preclinical models, presents a significant societal issue.

CHAPTER II

AN INTERGENERATIONAL CROSS-FOSTERING STUDY OF THE EFFECTS OF COCAINE ON MATERNAL BEHAVIOR AND AGGRESSION

Introduction

As discussed in the general introduction above, maternal cocaine abuse during pregnancy has been associated with deficits in maternal-infant "bonding" (Burns *et al.* 1991), and mothers with a history of drug abuse often exhibit poor mother-infant interactions (Bauman et al. 1983; Bays 1990; Howard et al. 1995; Johnson et al. 1990). Though studies with human subjects are helpful in understanding the connection between cocaine-use and maternal neglect, these experiments are correlational at best. There is an unavoidable lack of control over many important variables that could confound the results, such as socioeconomic issues, lack of family support, multi-drug abuse, and poor general prenatal care (Chasnoff *et al.* 1998; Koren *et al.* 1998). However, despite this lack of control, studies that employ numerous controls have shown a strong correlation between reported history of child maltreatment and the perpetration of maltreatment and/or neglect in next generation mothers (Egeland *et al.* 1987; Hunter *et al.* 1978).

In order to appropriately investigate and describe the characteristics of cocaine-induced disruption of maternal behavior and potential neglect, as well as possible intergenerational effects of such disruptions, a non-human cocaine abuse model offers several advantages. The laboratory rat is a particularly good model for the study of maternal behavior. Their offspring are born blind, unable to thermoregulate, defecate, urinate, or protect themselves from attack (Numan 1994), thus needing considerable maternal care to survive (Stern 1997). Behaviorally and neurologically, maternal behavior in the rat has also been relatively well characterized (Numan 1994; Pedersen *et al.* 1982;

Pedersen *et al.* 1994) so that any insult to normal maternal behavior can be easily determined. One subset of maternal behaviors in the rat is maternal aggressive behavior, related to protecting offspring from intruders into the nesting environment (Gammie 2005; Numan 1994). Maternal aggression is found in most mammals and has been characterized as an offensive/aggressive series of actions and postures, including direct attacks on an intruder, thought to help ensure offspring survival (Numan 1994). Maternal aggression can be elicited during the late gestational period, but is thought to peak during the first 10 postpartum days (PPDs) (Giovenardi *et al.* 1997).

The results of the animal studies to date seem to replicate the disruptions in maternal care seen in human populations. There is general agreement that acute cocaine treatment in rat dams disrupts both early onset and established pup-directed maternal behavior, while increasing locomotor behavior and stereotypies (Johns et al. 1994c; Johns et al. 1998b; Kinsley et al. 1994; Zimmerberg et al. 1992). Significant disruptions in maternal behavior following chronic gestational cocaine treatment during pregnancy were reported only for the onset of maternal behavior or very early postpartum period, and these dams did not display the hyperactivity often seen in acutely treated dams (Heyser *et al.* 1992; Johns *et al.* 1994c; Kinsley *et al.* 1994; Peeke *et al.* 1994; Vernotica *et al.* 1996a). To our knowledge, no reports have been previously published on the intergenerational effects of such treatment on maternal behavior; however, numerous studies have demonstrated the non-genomic intergenerational transmission of naturally occurring variations in maternal behavior and stress responsivity (Champagne et al. 2001b; Francis et al. 1999; Meaney 2001; Weaver et al. 2004).

In addition to its effect on maternal behavior, drug abuse has long been associated with both anxiety and aggressive behavior (Moss et al. 1993). Research from several labs has reported that chronic and acute cocaine-treatment can both alter maternal aggression, sometimes in a dose-dependent fashion (Heyser *et al.* 1992; Johns *et al.* 1994c; Johns *et al.* 1998b; Lubin *et al.* 2001; Vernotica *et al.* 1996a). Chronic cocaine-treatment has been shown to increase maternal aggression significantly by PPD six (Johns *et al.* 1994c), and under certain conditions, PPD 10 (Heyser *et al.* 1992), while acute treatment has been shown to decrease it (Johns *et al.* 1994c; Johns *et al.* 1998b;

Vernotica *et al.* 1996b). Importantly, the effects of chronic cocaine-treatment on aggression do not result from cocaine withdrawal (Johns *et al.* 1997b). Most findings to date are reported for lactating dams during the earlier postpartum period at more moderate doses of cocaine, with some data available for the later postpartum period at higher doses (Heyser *et al.* 1992). Interestingly, the concept of intergenerational transmission of stress responding through altered maternal behavior has gained attention over the last few years (Champagne et al. 2001b; Fish et al. 2004; Francis et al. 2000; Francis et al. 2002; Pedersen et al. 2002). To our knowledge there are no reports of intergenerational studies examining maternal aggression following cocaine treatment or exposure in the later postpartum period.

This initial study was designed to examine if the previously reported effects of cocaine-treatment on maternal behavior and maternal aggression persist into the next generation, and if these effects were primarily related to the offspring prenatal exposure condition, maternal care experience, or the interaction between both of these factors. We hypothesized that the onset of maternal behavior in first generation dams (FGDs) would be altered primarily by prenatal cocaine exposure, with secondary effects resulting from the maternal care environment, and the largest behavioral effect resulting from the combined effect of poor maternal care and prenatal exposure to cocaine. With regards to cocaine's effect of maternal aggression, we hypothesized that the cocaine-induced increases in maternal aggression previously reported on PPD six (Johns *et al.* 1994c; Johns *et al.* 1998b) would continue in mothers at later points and would affect the first generation offspring's later aggressive behavior. In the first generation dams, we expected a lesser, but still elevated level of maternal aggression resulting primarily from prenatal cocaine exposure, with a lesser effect due to the rearing environment, and the strongest effect seen in animals both exposed to cocaine and reared by cocaine-treated mothers.

Methods

Subjects:

Following a two-week habituation period, virgin female (200-240 grams) Sprague-Dawley rats (Charles River, Raleigh, NC) were placed with males on a breeding rack until a sperm plug was found, which was designated as gestation day (GD) zero. Subjects were randomly assigned to treatment or control groups and singly housed and maintained on a 12:12 reverse light cycle (lights off at 0900) for seven days. They were then transferred to a room with a regular light cycle (lights on at 0700) for the remainder of the experiment, a procedure that generally results in the majority of dams delivering their litters during daylight hours (Mayer et al. 1998).

While a total of five treatment groups were presented in the original publications (Johns *et al.* 2005a; McMurray *et al.* 2008b), for the simplicity of our discussion the groups have been reduced to the following: chronic cocaine (CC), chronic saline (CS), and untreated (UN) dams. Chronic cocaine and CS dams received subcutaneous injections twice daily throughout gestation (GD 1-20) on alternating flanks, of 15 mg/kg cocaine HCL (dose calculated as the free base; Sigma Chemical Company, St. Louis, MO) dissolved in 0.9% normal saline (total volume 2mg/kg), or normal saline (0.9%) respectively, at approximately 8:00 am and 4:00 pm. UN dams were weighed and handled daily but received no drug treatment. All treatment groups had free access to water and food (rat chow), except the CS treated dams who were pair fed to match CC dams in order to control for the anorectic effects of cocaine, as previously described (Johns *et al.* 1994c). Separate groups of animals were designated for maternal behavior and maternal aggression testing. A schematic outline of the testing schedule for each group can be found in Figure 3. All procedures were conducted under federal and institutional animal care and use committee guidelines for humane treatment of laboratory subjects.

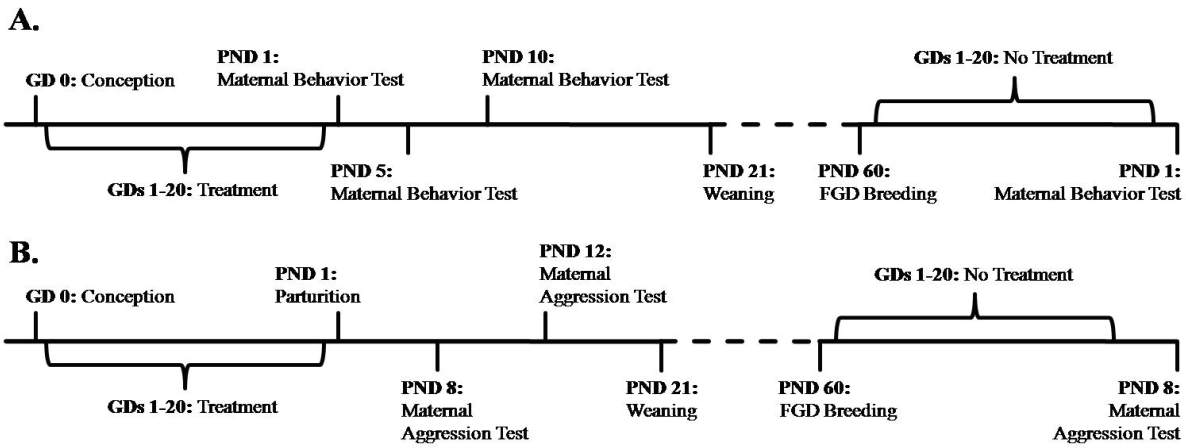

Figure 3. Timeline of intergenerational testing. (A) maternal behavior test; (B) maternal aggression test.

Cross-fostering

On the day of parturition, pups were removed from each dam, weighed, counted and their gender determined before being culled to a litter of 4 males and 4 females. Litters were culled 30 minutes before testing and then returned to either their natural mothers at time of testing or the entire litter fostered to a dam from a different treatment or control group having delivered as closely as possible to the same time (usually within several hours) who had her own pups removed. Dams and their litters were matched for delivery time and cross-fostering interval in all groups. Group numbers varied somewhat because of the loss of some animals during testing and the necessity of breeding extra dams to deliver at specific times to allow fostering of specific groups. Cross-fostering allowed for independent assessment of the effects of prenatal drug exposure of a rearing litter and the effects of maternal drug treatment (or the interaction of these conditions) on maternal behavior and maternal aggression in original test dams. We were also able to determine the effects of prenatal drug exposure, as well as the effects of treatment condition of the rearing mother on the maternal behavior of first generation offspring dams.

Original Dam Maternal Behavior Testing

The procedure for maternal behavior testing has been previously described (Johns *et al.* 1994c). Following delivery of their final pup, dams and their litters were brought in their home cage

to an enclosed behavioral observation room, 400cm x 460cm, where dams were removed from their cage and weighed, and their pups removed. Dams were placed back in their home cages without pups, and the cages placed in a 60.96cm x 40.64cm x 50.80cm dimly lit testing cubicle, designed to reduce environmental distractions, for a 30-minute habituation period. During the habituation period, litter measurements were taken and litters were culled to four female and four male pups. Then, either her natural litter or a foster litter culled at the same time was placed in a warm, (room temperature) plastic cage lined with paper towels on top of the dam's testing cubicle. After habituation, nesting material (10, 2.54cm strips of paper towel) was placed at the back of the cage and each dam's culled litter (fostered or natural) was placed in the front of her cage. Videotaping with a Panasonic VHS (AG188U) recorder with low light sensitivity began as soon as the pups were placed in the cage and continued for 30 minutes on PPD one. After testing, dams and their culled litters were returned to the colony room until the next testing session (PPD five). On PPDs five and 10, the testing procedure for maternal behavior was the same, excepting that after PPD one, each dam kept her litter assigned on PPD one throughout the entire study until weaning, and maternal behavior was only recorded for a 15-minute period. We reduced the test time after PPD one because we found in pilot studies that group differences were still apparent with a 15-minute test period, and that scoring all sessions for 30 minutes was very labor intensive and thus prohibitive. At the end of each maternal behavior test, dams and their culled litters were returned to the colony. Dams remained with their pups until weaning on PPD 21. Recorded sessions from PPD one, five, and 10 were later analyzed for frequency, duration, and latency of the following 11 behaviors: **nest-build** (dam manipulates or moves the paper strips with her mouth or paws), **touch/sniff pups** (dam touches pups with her nose or front paws or sniffs them), **retrieve 2 pups** (dam has carried 2 pups from the front to the rear of the cage), **self-groom** (dam grooms herself with her tongue or paws), **rest off/lie on** (dam rests away from the pups or lies flat on top of them in a non-feeding position), **crouch** (dam stands over the pups with back arched in the nursing position, legs stiff and straight and head lowered), **retrieve 6 pups** (dam has carried 6 pups from the front to the rear of the cage), **lick pup** (dam licks a pup), **retrieve 8**

pups (dam has carried all 8 pups from the front to the rear of the cage), **rear-sniff** (dam rears on hind legs and sniffs the cage or air), and '**other'** (any behavior other than those designated above).

Original Dam Maternal Aggression Testing

Maternal aggression was assessed using a procedure similar to one previously described (Johns *et al.* 1998b; Lubin *et al.* 2003). Tests were conducted on PPDs eight and 12 for the original dams. On the morning of PPD eight, between 0800 and 1100 hrs (during the light phase), dams and their litters were brought in their home cage to a behavioral observation room where weight gain was recorded. On both test days, all dams and litters habituated to the test area in a quiet environment for 25 minutes, after which the home cage was placed in a 61x41x51cm dimly lit testing cubicle, designed to reduce environmental distractions, for a further 5-min habituation period. A smaller intruder male (~175 grams) was then placed in the front of the cage and the session was videotaped with a low light sensitivity VHS recorder for 10 minutes, after which the session was terminated. A new inexperienced intruder male was used for each session with no male used more than once. If the pups were attacked by the dam or the intruder, or if the dam or intruder was severely injured, the session was immediately terminated and data was excluded from analysis. Terminated sessions were noted for each group. After testing on PPD eight, dams and their culled litters were returned to the colony room and monitored for health daily. Maternal aggression was again assessed on PPD 12, using the same procedures as on PPD eight.

Observed maternal aggressive behaviors included maternal behaviors, defensive behaviors (threat), aggressive behaviors varying in intensity (rough groom, nip/bite male, aggressive posture, fight attack) and general activity. These behaviors of interest have been described previously (Johns *et al.* 1998b; Lubin *et al.* 2003) and included: **maternal behavior** (dam licks pups, moves pups, or crouches over pups); **rough-groom** (dam grooms intruder male roughly, usually around head, neck or back); **lateral / front threat** (dam threatens male while approaching with her body in a lateral position, or moves her face close to the males face often accompanied by teeth chattering); **fight /**

attack (dam makes a quick lunge usually followed by rolling, biting, and fur pulling directed towards the neck and back regions of the intruder); **nip / bite** (dam nips or bites male; differentiated from a fight attack by degree and lack of jumping and lunging); **aggressive posture** (dam forces intruder into a full submissive posture and pushes him down with extended front paws or stands over him with her paws on his chest or belly); and **general activity** (including general locomotor behaviors and other non-aggressive motor activities). Dams and litters were returned to the colony room after testing on PPD 12 and pups were weaned and separated on PPD 21.

First Generation Dam Subjects and Testing

After weaning on PPD 21, litters were separated by sex into same-sex housing (4 males/females per cage), from which one female from each litter was randomly selected at 60 days of age for breeding and testing for the onset of maternal behavior on PPD one or maternal aggression on PPD eight. Remaining pups from the litters were used for other behavioral tests at various ages (not reported here). Breeding conditions were the same as with the original treatment dams except that all animals were drug-naive, were tested with their natural litters, were bred to different males, and were fed ad libitum. FGDs were assigned group designations based on their prenatal exposure condition and their rearing dams' drug treatment (for example, CCCS indicates that the dam that reared the first generation dam was CC treated but the dam she was born to was treated gestationally with CS, thus she was prenatally exposed to saline). First generation dams were weighed every five days to monitor weight gain throughout pregnancy.

On the day of expected delivery, first generation dams were monitored throughout the day until delivery of their final pup, at which time their natural litters were culled to four males and four females for maternal behavior testing. Testing procedures for first generation dams were essentially the same as for the original parent dams on PPD one, except there was no cross-fostering. At the end of the 30-minute behavioral test, dams and their culled litter were returned to the colony. Offspring from mothers tested for maternal aggression were tested for maternal aggression with their own

culled litter on PPD eight using testing procedures as described above, except that they were tested with eight of their own pups that they had reared from delivery (four male and four female). At the completion of the 10-min behavioral test, dams and their litters were returned to the colony.

Data Analysis

Videotaped sessions were scored by two independent observers blind to treatment condition with inter-and intra-reliability set at 95-100% concurrence for frequency and latency, and 80% or better for duration of behaviors displayed by the dam using a computer program that calculated the frequency, duration, and latency of all relevant behaviors displayed by the dams. Behaviors not displayed by the dam were assigned a frequency and duration of 0 and the highest possible latency (1800 seconds for a 30-minute test, 900 seconds for a 15-minute test).

For statistical analysis of maternal behavior data, two-factor (drug treatment x litter prenatal exposure) Analyses of Variance (ANOVA) for between groups were employed for original dams and first generation offspring (prenatal exposure condition x rearing dam treatment) on PPD one. Test durations for PPDs five and 10 were only 15 minutes, so the data could not be directly compared to PPD one. Repeated measures ANOVA were employed for original dams on PPDs five and 10 for between and within group differences (drug treatment x litter prenatal exposure). Tukey (HSD) tests were used for post hoc analyses and statistical significance was set at less than or equal to the 0.05 level with relevant trends acknowledged in the results or discussion. Effects on maternal behavior of original dams as a result of drug treatment across PPDs, effects based on their rearing litter condition (litter prenatal exposure), and interaction effects (dam treatment by prenatal litter condition) are presented. Effects on first generation dam maternal behavior are presented based on prenatal exposure condition, rearing condition (treatment of rearing dam), and interaction effects (rearing by prenatal).

For statistical analysis of maternal aggression data, due to the relatively low levels of many behaviors, standard ANOVAs could not be used (data were non-normal). Thus, generalized linear models were used (Zeger et al. 1986), allowing for the usage of non-normal distributions, and

resulting in a series of test statistics with a Chi-squared (χ^2) distribution under the null hypothesis (for application review, see (Hanley *et al.* 2003)). Repeated measures log linear models for count data (frequency) were used to examine between group differences within each day as well as over repeated days of testing. Repeated measures weighted additive models for time to event best fit the duration dataset. Weights were inversely proportional to the within-cell (rearing, prenatal, session) variance estimate. Latency data were analyzed using the Cox Proportional Hazard model, a semi-parametric survival analysis procedure. To account for multiple observations in each rat, general estimating methods were used to obtain group estimates and standard errors, and p-values were adjusted for multiple comparisons via the FDR method (Benjamini *et al.* 2001). Estimates of the mean and standard errors under the model are presented graphically for frequency and duration data. Statistical significance was set at the $p \leq 0.05$ level. Following the original dam treatment effects, results based on the dam's foster-litter prenatal exposure condition are presented, and finally any effects resulting from the combined treatment and rearing litter interaction are presented. Results for first generation dams are first listed as those resulting from only their rearing dam's treatment (drug treatment of their rearing dam), followed by prenatal exposure effects (treatment of their biological dam), regardless of their rearing condition, and finally results based on the interaction of both rearing and prenatal environment. Given the large amount of data, only statistically significant results are presented in text.

Results

Original Dam Gestational Variables

There were significant effects of dam treatment on gestational weight-gain [$F(4,354)=20.95$, $p\leq0.01$] and litter birth weight [$F(4,360)=2.75$, $p\leq0.03$], with cocaine-treated dams displaying reduced weight gain over the gestational period ($p\leq0.05$), and cocaine and saline-treated dams displaying reduced litter birth weight compared to untreated dams ($p\leq0.05$). Lower litter birth weight in the CC and CS treated dams may be the result of either cocaine treatment or stress, but may also be related to a non-significant reduction in litter size in these treatment groups.

Original Dam Maternal Behavior

On PPD one, there were significant effects of dam treatment on the duration of crouching [$F(4,331)=4.24$, $p\leq0.01$], nest-building [$F(4,332)=4.12$, $p\leq0.01$], and self-grooming [$F(4,333)=2.65$, $p\leq0.03$]. There were also significant effects on the frequency of nest-building [$F(4,333)=2.59$, $p\leq0.04$], self-grooming [$F(4,333)=3.51$, $p\leq0.01$], rear-sniff [$F(3,333)=2.71$, $p\leq0.03$], and 'other' [$F(4,333)=2.57$, $p\leq0.04$], and on the latency to begin nest-building [$F(4,333)=3.28$, $p\leq0.01$]. CC treated dams crouched less (duration $p\leq0.01$) than both CS and UN treated dams, and had a longer duration of ($p\leq0.03$) and shorter latency ($p\leq0.02$) to nest-build than UN treated dams. CC treated dams also spent more time performing non pup directed behaviors (self-groom, other, rear/sniff) than CS and UN treated dams ($p\leq0.01$).

There were also significant between group main effects of dam treatment on the frequency [$F(4,321)=2.72$, $p\leq0.05$] and latency [$F(4,321)=3.11$, $p\leq0.05$] of nest-build, and on the duration [$F(4,321)=2.77$, $p\leq0.01$] and latency [$F(4,321)=2.38$, $p\leq0.05$] of self-grooming on PPD 5. There were also significant between group effects of dam treatment on the frequency [$F(4,321)=2.72$, $p\leq0.05$] of nest-building and duration of self-grooming [$F(4,321)=2.77$, $p\leq0.01$] on PPD 10. There was a significant within group main effect of dam treatment between PPD 5 and 10 on crouch frequency [$F(4,321)=4.77$, $p\leq0.01$] and duration of self-groom [$F(4,321)=2.77$, $p\leq0.05$]. Untreated and

chronically treated groups crouched more often (but for shorter durations) on PPD 5 than 10 and while activity was generally constant, self-grooming was increased in intermittent groups on PPD 10 compared to 5.

The prenatal exposure condition of the litter significantly affected the duration of nest-building by all dams [$F(4,333)=2.52$, $p\leq0.04$]. Dams that reared pups prenatally exposed to CC spent less time nest-building compared to dams that reared pups prenatally exposed to CS ($p\leq0.02$). There were also many strong trends that persisted over the entire testing period (PPD one through 10) indicating that CC pups received less overall maternal care (less or later crouching, licking, touching, more resting away from pups) than offspring from other prenatal exposure conditions.

There was a significant interaction between dam treatment and litter exposure on the latency to crouch [$F(16,332)=1.86$, $p\leq0.02$]. CC-treated dams rearing CS or UN exposed pups crouched later than all other chronically treated dams rearing pups from any prenatal exposure group ($p\leq0.05$). There was also a significant interaction of dam treatment and prenatal litter exposure on within-group lick pup latency [$F(16,321)=2.27$, $p\leq0.01$] across PPDs five and 10. All groups licked later on PPD 5 than 10 except CCCS, UNCC, and UNCS groups ($p\leq0.05$).

Original Dam Maternal Aggression

There were no differences resulting from dam treatment on defensive behaviors (threat), maternal behaviors, aggression, or general activity on either PPD eight or 12. Levels of most behaviors were relatively low in all groups compared to those previously reported (Johns *et al.* 1994c) at earlier times during the postpartum period (PPD 6). There were also no effects of litter prenatal exposure condition on defensive, aggressive, maternal behavior, or general activity levels in dams, and no effects resulting from the interaction of dam treatment and litter prenatal exposure.

First Generation Dam Gestational Variables

There were no significant effects of rearing condition or prenatal exposure condition on any gestational measure.

First Generation Dam Maternal Behavior

There was a significant main effect of rearing condition on the frequency of 'other' behaviors [$F(4,262)=2.72$, $p\leq0.03$]. First generation dams reared by CC-treated dams performed 'other' behaviors less frequently than CS reared dams ($p\leq0.01$) and had a non-significantly lower frequency of crouching [$F(4,260)=2.32$, $p=0.06$] compared to UN and CS reared dams.

There were also significant effects of prenatal exposure on the latency to retrieve all 8 pups [$F(4,262)=3.25$, $p\leq0.01$] and the duration of rest away/lie on pups [$F(4,264)=2.79$, $p\leq0.01$]. CC exposed dams took longer to retrieve all eight pups than CS ($p\leq0.01$) and UN (ns) exposed FGDs, and spent more time resting away from pups or lying flat on top of pups than did CS or UN exposed dams ($p\leq0.01$).

There was a significant interaction effect on the latency to touch/sniff pups [$F(16,262)=1.86$, $p\leq0.02$]. First generation dams with no prenatal drug exposure that were reared by CC treated dams (CCUN) touched pups later than FGDs from any other rearing condition ($p\leq0.05$) and later than any other CC-reared FGDs from any prenatal exposure condition ($p\leq0.05$).

First Generation Dam Maternal Aggression

FGDs reared by cocaine-treated dams, regardless of their prenatal exposure condition, exhibited a number of behavioral effects related to rearing condition alone. CC-reared FGDs had higher frequencies of aggressive posture and rough grooming of the intruder compared to CS-reared FGDs (aggressive posture, $\chi^2_{(1)}=10.76$, $p\leq0.01$; rough groom, $\chi^2_{(1)}=5.51$, $p\leq0.02$). Conversely, they had significantly lower levels of nip/bite compared to both UN- ($\chi^2_{(1)}=10.14$, $p\leq0.02$) and CS-reared ($\chi^2_{(1)}=4.57$, $p\leq0.03$) FGDs.

Prenatal exposure to chronic cocaine, regardless of rearing condition, also resulted in behavioral differences in first generation dams. CC-exposed FGDs exhibited maternal behaviors less frequently ($\chi^2_{(1)}$=3.92, p≤0.05, see Figure 7) and were more frequently defensive and aggressive than CS-exposed control FGDs (threat, $\chi^2_{(1)}$=4.59, p≤0.04; aggressive posture, $\chi^2_{(1)}$=7.23, p≤0.01).

FGDs prenatally exposed to chronic gestational cocaine and reared by their own CC-treated dams (CCCC) displayed fewer instances of maternal behavior than control FGDs (UNUN, $\chi^2_{(1)}$=7.26, p≤0.02; CSCS, $\chi^2_{(1)}$=5.89, p≤0.02). They also displayed a higher frequency of aggressive behaviors than CSCS FGDs (rough groom, $\chi^2_{(1)}$=6.92, p≤0.01; aggressive posture, $\chi^2_{(1)}$=17.62, p≤0.01).

Discussion

Maternal Behavior

The findings of the maternal behavior study support our hypotheses, as cocaine drug exposure as well as differential maternal treatment disrupted the onset of maternal behavior in first generation offspring. The effects on the original dams have been reported (Johns *et al.* 2005a), but to briefly summarize, the disruptive effects of cocaine treatment on PPD one, regardless of litter prenatal exposure, are similar to those previously reported with cocaine-treated dams rearing only surrogate offspring (Johns *et al.* 1994c). In addition to the decreased crouching seen here, consistent with previous reports following cocaine treatment, there were also non-significant trends for these dams to touch pups later, rest away from or lie flat on pups longer, and to lick pups later than other dams, indicating a general disruption of pup directed behavior on PPD one with non-significant trends continuing to PPD five. A slight increase in activity-related behaviors of cocaine-treated dams on PPD one was also found in this study, but had not been previously reported in dams that did not have cocaine in their bloodstream at the time of testing.

More applicable to our focus on first generation offspring were the effects of litter prenatal exposure on the original dam behavior and consistent trends across the postpartum period. Similar to prior reports of cocaine-treated dams rearing surrogate pups, in this study cocaine-treated dams rearing control (saline and untreated) pups began crouching later than other dams (Johns *et al.* 1994c; Johns *et al.* 1994b; Johns *et al.* 1996; Johns *et al.* 1998c; Kinsley *et al.* 1994; Vernotica *et al.* 1996a). In a design similar to the one used here, Heyser and colleagues reported that the maternal behavior of cocaine-treated dams did not differ from controls when examined in later postpartum periods (PPDs five through nine) and when rearing either their own biological offspring or fostered offspring (Heyser *et al.* 1992). Here, we report similar findings with respect to diminishing group differences later in the postpartum period; however, we also unexpectedly found a pattern of differential treatment of pups prenatally exposed to cocaine. This effect was shown by all dams, regardless of the dam's own treatment condition. Some significant effects and many consistent trends, some quite

strong considering the design ($p \leq 0.06$-0.07), indicating impaired or delayed nesting, less crouching, licking, touching of pups, and more time spent resting away from pups or lying flat on pups that were prenatally exposed to cocaine. In our subjects, the behavior of lying flat on the pups did not appear to stimulate nursing, although others may disagree (Stern et al. 1989).

Rat pups prenatally exposed to cocaine differ in their stress responsivity, ability to elicit play solicitations from an untreated conspecific (Johns et al. 1995; Overstreet et al. 2000), have different activity levels, and some early physical developmental differences such as lower body weight, smaller head circumference, and potentially different cry patterns (Johns *et al.* 1992b; Johns *et al.* 1992a). These data taken together suggest that behavior and physical attributes of drug exposed offspring may make them more vulnerable to neglect or even abusive behavior. Preliminary data in humans suggests that premature babies or babies with low birth weight are physically unattractive and emit disturbing high pitched, arrhythmic cries leaving them more susceptible to abuse and neglect (Belsky 1993; Brunk et al. 1984). The specific attributes of offspring with prenatal cocaine exposure that may lead to poorer social outcomes is potentially a very interesting topic.

First Generation Dams

Our hypotheses concerning the maternal behavior of the FGDs were supported in that we did find both rearing and prenatal effects on the onset of maternal behavior, with more significant effects in the FGDs prenatally exposed to cocaine. The effects of prenatal cocaine exposure in FGDs were different from the effects of rearing condition in several ways. Retrieval and time spent away from pups were altered in the cocaine-exposed FGDs, who also demonstrated non-significantly shorter durations of crouching, touched pups significantly less, nest-built less, and were more active than other FGDs. These effects are somewhat reminiscent of the cocaine-treated original dams, in terms of activity levels and crouching, but to a much less extensive degree. Alternatively, rearing by cocaine-treated dams, regardless of prenatal exposure condition, disrupted the frequency, but not duration, of crouching in FGDs, and they were generally less active; a series of effects strikingly similar to their

rearing dams (see Figure 4). Interestingly, they also exhibited trends to touch, crouch, and lick pups later, as well as rest away from pups more often than other FGDs from different rearing conditions. This was the first systematic investigation reporting effects of cocaine exposure and rearing condition on mothers and their offspring.

The fact that despite the cocaine treatments, the drug treated dams performed relatively adequately maternal behavior, especially in the later postpartum period, highlights the robust nature of maternal behavior in rats. Although the rearing effects were not particularly robust effects, they likely impact other types of offspring behavior (other than maternal), particularly in combination with effects of prenatal exposure to cocaine. Given the relatively mild effects we see in rat dams compared to the effects reported in human populations, these findings indicate factors such as polydrug abuse and environment probably play a larger role in behavioral differences, and that future animal studies focusing on the interaction between stress, environment, and relevant polydrug abuse models may be very informative. One important common finding in this and other intergenerational studies (cocaine or maternal separation) is that there is some transfer of behavior resulting from altered maternal care, which manifests itself in the next generation.

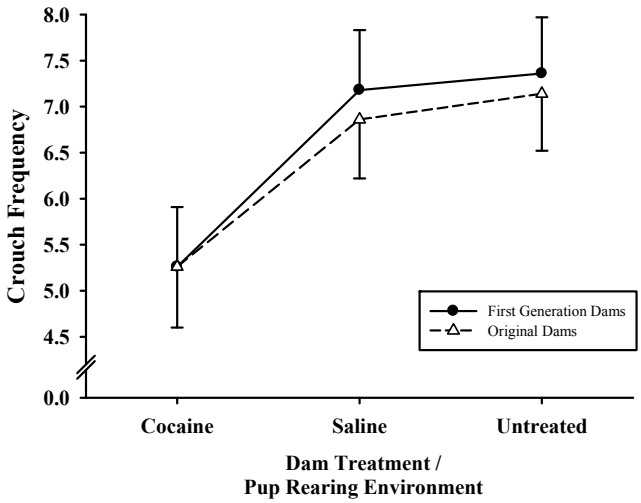

Figure 4. The relationship between maternal behavior received and maternal behavior performed.

Several conclusions may be drawn from the maternal behavior study including: a) that chronic treatment alters primarily the onset of maternal behavior in rat dams (when cocaine is not in their system), which diminishes across the postpartum period; b) that there are intergenerational effects of cocaine on the onset of maternal behavior in FGDs associated with prenatal cocaine exposure, rearing condition, or the interaction of these two factors, but to a lesser degree than those

seen in the original cocaine-treated dams; and c) that prenatal exposure to cocaine increases the likelihood that a litter will receive less pup-directed care compared to controls.

Maternal Aggression

Using this paradigm we did not find that cocaine-treated dams exhibited higher levels of maternal aggression towards intruders on PPD eight as we had hypothesized. Historically, significant increases in maternal aggressive behavior in gestationally treated dams rearing surrogate pups have been seen on PPD six through ten (Johns *et al.* 1994c; Johns *et al.* 1997a; Johns *et al.* 1998b; Johns *et al.* 1998a). When higher doses of cocaine are used, these effects can extend through PPD twelve (Heyser *et al.* 1992), however behavioral effects apparently diminish by PPDs eight through 12 using the dose and regimen used here. Without testing throughout the entire postpartum period, we cannot say whether the early increased levels of aggression are a truly transient effect. Importantly however, our findings may also be a factor of the different cross-fostering conditions in this study, as previous reports used untreated surrogate pups, and as indicated in the maternal behavior study, dams rearing cocaine-exposed pups exhibit a number of altered behavioral patterns.

During aggression testing, we rarely see high rates of pup-directed maternal behavior, as the dam is generally more focused on the intruder than in performing these behaviors over the short test period. Thus, the lack of group differences in crouching, licking, and touching of pups during testing was not surprising. Along with maternal behavior, the frequency and duration of all behaviors examined were similar on both PPDs eight and 12, with slightly lower overall maternal aggression levels on PPD 12, consistent with prior reports that maternal aggression diminishes after PPD 10 (Flannelly et al. 1987; Mayer et al. 1987).

First Generation Dams

Regardless of their prenatal exposure condition, FGDs reared by cocaine-treated dams, exhibited increased maternal aggressive behavior, the type and degree largely dependent on their

rearing dam's drug history. FGDs reared by cocaine-treated dams were more likely to pin intruders and have direct contact with the male, but were less likely to nip/bite the male than were controls. The differentiation of the type and degree of aggressive behaviors in the next generation is perhaps something that needs to be studied further, as it may provide clues to the mechanisms through which cocaine is be acting. As seen in the maternal behavior study, the less than optimal maternal behavior received when pups are reared by cocaine-treated dams appeared to have influenced behavior of the FGDs. Perhaps these findings reflect the strong link between rearing environment and behavior as suggested in numerous published reports of the non-genomic transmission of behavior (Champagne et al. 2001b; Fish et al. 2004; Francis et al. 1999; Meaney 2001; Pedersen et al. 2000).

The behavioral effects of prenatal exposure to cocaine have been reported in numerous papers, too extensive to describe here. However, for the purposes of this discussion it is important to note that prenatal cocaine exposure in rat pups has been shown to alter stress responsivity in adolescence and adulthood (Bilitzke et al. 1992; Campbell et al. 2000; Molina et al. 1994; Planeta et al. 2001; Spear 1996; Wood et al. 1995), play behavior in adolescence (Wood *et al.* 1994; Wood *et al.* 1995), the ability to elicit play or social interactions (Johns et al. 1995; Overstreet et al. 2000), and social/aggressive behavior at older ages (Johns *et al.* 1994b; Overstreet *et al.* 2000). Most of the literature to date has focused on resident intruder and social aggression in males; however, maternal aggression and female aggression differ from male aggression in a number of respects, and thus generalizing across paradigms may be unwise.

Given this literature though, we expected to see more aggression in our offspring. FGDs that were prenatally exposed to cocaine did exhibit minor increases in several aspects of maternal aggression, regardless of their rearing environment (rearing dam treatment). It is important to note that cocaine-exposure was associated with higher levels of aggression than was rearing by a cocaine-treated dam. The more aggressive behavior of cocaine-exposed FGDs we report here, although less intense than other types of non-maternal aggression seen in male offspring (Johns et al. 1994b; Johns et al. 1995; Wood et al. 1998), establishes aggression as a potential behavioral target of prenatal

cocaine exposure in both sexes. As we expected, the FGDs exposed to cocaine and also reared by cocaine-treated dams were the most aggressive and least maternal offspring, highlighting both the individual and interactive effects of prenatal and rearing conditions.

There were several instances of behaviors in saline-reared (rough groom, aggressive posture) or exposed (threat, nip/bite, aggressive posture) FGD groups that were performed even less frequently than in UN-reared or unexposed FGDs. Although we have no evidence to support stress related effects in this group, we believe this is very likely an important factor, and indicates a need for further research on the effects of stress alone on these behaviors.

Regarding the limitations of the current study, cross-fostering alone has been shown to result in many behavioral alterations (Francis *et al.* 1999), but these effects were offset by ensuring that all dams received litters from another dam, generally within an hour of final pup delivery. The ability to isolate these two potential individual factors (prenatal exposure and rearing environment) in relation to maternal behavior and aggression in both dams and offspring was a significant strength of this study, and indicates the importance of examining the separate contributions and the interaction of both mother and offspring in response to drug treatment. The finding that infants prenatally exposed to cocaine are differentially treated by all dams indicates that something is different about these pups, perhaps making them less able to elicit normal care. This idea, combined with the knowledge of the importance of maternal care on future offspring development, helped to determine our future research aims concerning how these pups are different.

CHAPTER III

AN INVESTIGATION OF PRENATAL COCAINE'S EFFECT ON EARLY POSTNATAL THERMOGENESIS AND VOCALIZATION PRODUCTION

Introduction

Unlike the prominent neuroanatomical and behavioral alterations seen following Fetal Alcohol Spectrum Disorder (Kodituwakku 2009; Norman *et al.* 2009), prenatal cocaine exposure is known to produce a myriad of subtle effects on developing infants. The behavioral effects of prenatal cocaine exposure are well documented (Bandstra et al. 2010; Chae et al. 2009) and a number of these effects may directly impact the ability of the infant to elicit optimal maternal care. Although deleterious effects of cocaine exposure on maternal stress responsivity and infant attention have been reported in human clinical populations (Strathearn et al. 2010a), as well as in studies of rodent mothers treated with cocaine while pregnant (Johns *et al.* 1998b; Johns *et al.* 2005a; Kinsley *et al.* 1994; McMurray *et al.* 2008b; Nelson *et al.* 1998; Quinones-Jenab *et al.* 1997; Vernotica *et al.* 1996a; Vernotica *et al.* 1999; Vernotica *et al.* 2007), the impact of cocaine on maternal and neonatal behavior is best viewed interactively (Eiden et al. 2011; Stern 1986). Thus far, few studies have systematically studied how cues from cocaine-exposed infants influence maternal care.

While changes in endocrine system function are a large determinant of the onset of maternal behavior (Keverne 1988; Numan 1994; Numan et al. 2003; Rosenblatt 1990), sensory input is also important for the onset and retention of maternal behavior (Morgan *et al.* 1992). A number of cues produced by infants can modify maternal responses, including vocalizations (Brunelli et al. 1994; D'Amato et al. 2005; Farrell et al. 2002b; Smotherman et al. 1974), odors (Levy *et al.* 2004), and temperature (Adels et al. 1986; Bates et al. 1985; Henning et al. 1982; Jans et al. 1990; Leon et al.

1985; Stern et al. 1996; Woodside et al. 1988). The importance of each cue depends loosely on the maternal environment, including the number of pups, age of the pup producing the cue, maternal experience, and the environment in which the nest resides (Brudzynski 2005; Champagne et al. 2001a; Champagne et al. 2003; Mattson et al. 2001). All of these behavior sets could affect maternal care and likely interact with any alterations in maternal stimulus perception and response resulting from potential drug exposure or other environmental disruption.

Thermoregulation presents a particularly interesting target for study, given both its own role in eliciting care as well as its involvement in other cues, such as ultrasonic vocalization production (discussed below). While human infant temperature regulation is largely accomplished in an individual manner, in rat pups thermoregulation is achieved on both a group and an individual level. In the early days of life, individual rat pups rely on external sources for heat production, such as their littermates and their mother, as well as internal metabolic sources. As a litter, rat pups achieve warmth through huddling, are insulated from cold by the nest, and receive additional heat from their mother during close contact nursing. Individual rat pups produce heat through a number of mechanisms, including shivering and locomotion to warmer environments, but primarily through brown adipose tissue (BAT) thermogenesis (Smith 1964). BAT thermogenesis is sympathetically driven by β-adrenergic activity, and once activated the mitochondrial uncoupling protein 1 effectively oxidizes fatty acids to produce heat. Brown adipose tissue is primarily located surrounding the heart and is also distributed throughout the peritoneum. Its location proximal to the heart is of particular importance, as the heart acts as a pumping mechanism to distribute the heat generated by BAT throughout the body via the circulatory system.

Generally, the volume of brown fat depends on the age of the animal, with older animals having significantly less brown fat than younger animals, in favor of white adipose tissue. Typically, as the rat ages alternative mechanisms of heat production and maintenance develop, such as better thermogenesis through locomotion and better thermoregulatory capacity as the pup increases in body size and thus insulative capacity (Spiers et al. 1986). Over development, BAT is transdifferentiated

into white adipose tissue, the primary purpose of which is to store energy in the form of triglycerides. Individual brown adipose tissue thermogenesis is immediately apparent at birth in rats and is of seminal importance to proper nervous system development (Blumberg 2002), may play a role in the development of obesity and diabetes (Cinti 2005; Cinti 2006; Cypess *et al.* 2009), and likely determines nursing opportunities. When in the huddle, heat is shared with littermates to reduce total heat loss and supplement maternally provided warmth, with BAT thermogenesis supplying additional heat to the litter (Alberts 1978). Rat dams are indeed capable of determining the current thermal state of pups, and their behavior is modified by their pups' thermal state (Adels et al. 1986; Bates et al. 1985; Henning et al. 1982; Jans et al. 1990; Leon et al. 1985; Stern et al. 1996; Woodside et al. 1988). Specifically, decreases in pup body temperature have been associated with increases in nursing behaviors in rat dams, during which maternal heat is passed to the litter.

The effects of prenatal cocaine exposure on pup thermal control have not been thoroughly investigated, although acute exposure to cocaine in adulthood does alter cardiac function in both humans and animals (Regalado et al. 1996; Sheinkopf et al. 2006a; Sheinkopf et al. 2007; Sun et al. 2003), and raises body temperature and impairs heat dissipation in humans (Crandall et al. 2002). Brown adipose tissue thermogenesis specifically is a likely target of prenatal cocaine exposure due to cocaine's direct impact on maternal norepinephrine systems as well as on the developing stress response systems of the fetus. Cocaine-induced prenatal malnutrition is also a likely contributor, as maternal cocaine use has been strongly associated with malnourishment of the mother and fetus, as well as placental vasoconstriction that further complicates nourishment delivery to the fetus. As is the case with fetal cocaine exposure, under- or over-provision of nutrients and even postnatal nutritional challenges can alter the long-term adipose tissue volume and function (Mostyn et al. 2009), potentially leading to obesity, diabetes, and disruptions in renal function (Cinti 2006).

Assessments of individual thermogenic ability in cocaine-exposed pups have not been studied previously, so it is unknown if this pup characteristic may be a contributing factor to the decreased maternal care of cocaine-exposed offspring. Any alterations in thermoregulatory capacity would

likely result in qualitative differences in vocalizing behavior. Thus, this study is designed to determine if there are differences in thermoregulation or ultrasonic vocalization resulting from prenatal cocaine exposure and if the normal relationship between these two behaviors is maintained. In humans, vocalizations have been used as important biomarkers of developmental delay, thus such findings in rodents would have great impact on predicting the level of insult caused by a drug.

In addition to its contribution toward the elicitation of maternal care, thermal state also has tremendous influence over vocalizing behavior in early life, which in turn contributes to the elicitation of maternal attention. Rats of various ages have been shown to vocalize in response to handling, cold temperatures, isolation, and social factors (Blumberg et al. 1992; Branchi et al. 2001; Hahn et al. 2005; Shair et al. 1997), thus vocalizations potentially constitute one major method of communication between rats (Brudzynski 2005; Brunelli *et al.* 1994). While the perceptual frequency range of human hearing is thought to be from 20 Hz – 20 kHz, rats are able to perceive frequencies up to 100 kHz. Additionally, rat pups are born able to vocalize at frequencies from 20 Hz – 100 kHz. Thus, along with vocalizations that occur in frequency ranges that are audible to humans, rats can vocalize in frequencies that would be ultrasonic to humans. For the purposes of this discussion, label ultrasonic vocalizations are considered relative to the human perceptual range: those produced with fundamental frequencies in the 20 kHz – 100 kHz range.

Similar to audible vocalizations, the complexity, rate, and duration of ultrasonic calls vary immensely with age and gender. At birth, vocalizations of any form rarely occur (Blumberg et al. 1996; Blumberg 2002). Those that do occur are thought to be a bi-product of a thermoregulatory mechanism called laryngeal braking. Blumberg and Alberts (1990) noted that ultrasound production by rat pups was remarkably similar to the audible grunting of human infants with respiratory distress syndrome (Blumberg et al. 1990), which are known to be an acoustic by-product of a respiratory mechanism called laryngeal braking. Laryngeal braking is a technique that literally brakes expiration to increase intrathoracic pressure, enhancing oxygen uptake in the lungs (Davis et al. 1987; England et al. 1985; Hofer et al. 1993), and thus providing additional oxygen. In infants undergoing

respiratory distress this provides much needed oxygen; however, laryngeal braking also boosts blood oxygenation to assist in brown adipose tissue metabolism and associated thermogenesis. This increase in intrathoracic pressure results in a comparatively quick expulsion of air from the lungs, which produces the vocalization as it flows past the vocal cords and out the respiratory tract. In humans, these vocalizations are typically audible; however, whether due to the diameter of the respiratory tract or properties of the larynx itself, rodent pups typically produce these in ultrasonic ranges. Vocalizations at this age are not thought to be socially intended by the pup producing the call, but are none-the-less an important indicator to the rat mother that the pup is in need of care.

As pups grow older, two changes typically occur that alter the production of vocalizations: the need for brown adipose tissue thermogenesis reduces in favor of more effective thermoregulatory mechanisms and the positive relationship between vocalizing and maternal attention is learned. Thus, the purpose of calling can become more socially mediated and less of an acoustic bi-product. This shift is thought to occur between postnatal day (PND) eight to ten (Blumberg et al. 2001), and such a shift in mechanism would suggest that characteristics of the call change as the pup develops. Indeed, ultrasonic vocalizations tend to decrease slightly in the fundamental frequency across the neonatal period as pups grow larger (Naito et al. 1987), while the duration of calling and the complexity of the sonographic characteristics of the call increase (Brudzynski *et al.* 1999; Brudzynski 2005). These developmental changes in vocalizations may in part regulate naturally occurring changes in maternal care over the postpartum period, and thus provide interesting targets to use in the study of developmental disorders.

Human vocalizations have been suggested as a marker for central nervous system integrity following prenatal insult; however, this relationship has not yet been established in rodents. Unfortunately, rodent studies have typically used poorly controlled thermal environments and have focused simply on the number of vocalizations emitted following removal from littermates and dam. These studies have shown some common themes though, mostly demonstrating decreased vocalizations following prenatal insult via drug exposure (Antonelli et al. 2005; Hahn et al. 2000;

Kehoe et al. 1991; Tattoli et al. 2001; Winslow et al. 1990) or malnutrition (Tonkiss *et al.* 2003). Unfortunately, few studies have been conducted longitudinally and even fewer studies have performed in depth analyses of assessed acoustic properties of the call. One such study examined prenatal pesticide exposure, finding a decrease in the duration of each call produced and an increase in the latency to emit the first call (Venerosi *et al.* 2009). Additionally, and perhaps most relevant to the current discussion, prenatal cocaine exposure has been shown to increase the starting pitch of calls following a mild thermal challenge, but that these effects were dependent upon the genotype of the subject (Hahn *et al.* 2000).

As a stimulus for maternal attention, a sustained high-rate of vocalizing by pups is the most effective for eliciting retrieval from dams (Brunelli et al. 1994; Deviterne et al. 1990; Farrell et al. 2002a; Fu et al. 2007; Zimmerberg et al. 2003) and can also be an important stimulus for the maternal consumption of pup excretions during anogenital licking (Brouette-Lahlou *et al.* 1992). In humans, alterations in infants cry patterns can illicit altered physiological and behavioral responses from their caregivers (LaGasse et al. 2005; Sheinkopf et al. 2006b; Tronick et al. 2005), though the direct mechanisms of these effects are still being revealed. In rats, it is thought that ultrasonic vocalizations may directly stimulate prolactin secretions in dams, thus altering maternal care, although some controversy exists (Hashimoto *et al.* 2001; Stern *et al.* 1984; Terkel *et al.* 1979). However, the implication of a direct effect of vocalizations on the endocrine systems of dams is certainly an interesting theory considering the altered endocrine system of drug-exposed dams.

It is likely that the effect of developmental exposure to drugs of abuse on ultrasonic vocalizations varies by the drug, developmental timing, and dose. Several studies reported alterations in ultrasonic vocalization following prenatal alcohol and cocaine exposure (Barron et al. 1996; Barron et al. 2000; Barron et al. 2005; Hahn et al. 2000; Kehoe et al. 1992); however, results following prenatal cocaine exposure alone are mixed (Meyer *et al.* 1996). Clearly, the study of vocalizations following prenatal cocaine bears great clinical relevance, as the cries of human babies with in utero cocaine exposure are perceived by mothers as more aversive than the cries of normal babies (Corwin

et al. 1992) and high-risk infants have shown altered patterns of vocalization (LaGasse *et al.* 2005). As discussed in Chapter 2, rat dams typically respond less or slower to cocaine-exposed pups, suggesting that something about these pups alters maternal motivation or ability to elicit normal levels of care.

Methods

Breeding

Individually housed 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 one week and then mated until conception was noted by the presence of a vaginal plug and sperm in a vaginal smear (gestation day (GD) 0). Following conception, females were randomly assigned to chronic cocaine, chronic saline, or untreated groups as they became pregnant (see below for treatment information). Weight gain was measured daily for all animals throughout gestation. Water and chow was available ad libitum for all except chronic saline-treated rat dams, who were matched with a chronic cocaine dam on a pair-feeding schedule to control for any effects of cocaine-induced anorexia. Seven days following conception (GD 7), females were moved to a colony room and individually housed on a regular 12:12 light:dark cycle with lights on at 7:00 AM. This procedure results in the majority of dams delivering in the normal daylight hours (Mayer et al. 1998). PPD 1 was defined as the calendar day during which delivery was completed. Following delivery, litters were culled to 10 pups (5 male, 5 female) and pups were returned to their own biological mothers. We chose not to employ surrogate mothers to control for differences in postnatal care between groups as we wished to study the more natural interactions of mothers and pups, as occurs in human clinical populations.

Dam Treatment

Females were randomly assigned to chronic cocaine, chronic saline, or untreated groups as they became pregnant, with 15 animals per group. Chronic cocaine-treated dams received twice-daily subcutaneous injections of 15 mg/kg of cocaine hydrochloride (total 30 mg/kg dose calculated as free

base, 2ml total volume, Sigma, St. Louis, MO) dissolved in normal saline at approximately 9:00 AM and 4:00 PM throughout gestation (GD 1-20) and not thereafter. This is the lowest chronic gestational cocaine treatment dose consistently reported to produce significant effects on pup-directed maternal behavior (Nelson et al., 1996). To prevent skin lesions, injections were alternated daily between rear leg flanks. If a lesion appeared, the fur was clipped at the site, cleaned daily with a betadine solution, and a topical antibacterial ointment (Polymycin-Bacitracin-Neomycin, E. Fougera & Co., Melville, NY) was applied to the area. These measures have been shown to minimize skin lesion appearance and severity and have been used in numerous studies (McMurray *et al.* 2008b).

Control groups included both chronic saline-treated and untreated dams. Chronic saline-treated dams received injections of normal (0.9%) saline (2ml/kg total volume) on the same schedule and regimen as the chronic cocaine dams. Saline-treated dams were pair-fed to chronic cocaine dams in the early gestation period such that the amount of food ingested by cocaine-treated dams on average on a specific gestation day during the first 7 days of gestation was the amount provided to saline-control dams on the corresponding gestational day. Beginning GD 8, saline-treated dams had free access to rat chow. This procedure accounts for the time when cocaine-treated dams are most affected by the anorectic effects of cocaine in prior studies (Johns *et al.* 2005a; McMurray *et al.* 2008b), while not inducing the serious confound of food deprivation in saline-treated dams. Untreated control dams received no drug treatment or food restriction during gestation or during the postpartum period, but were weighed daily to control for the effects of handling.

Apparatus, Temperature Measurement, and Vocalization Recording

The apparatus (shown in Figure 5) consisted of a double-walled glass chamber described previously (Blumberg et al. 1990; Blumberg et al. 1996). Temperature-controlled water was pumped between the walls to control the chamber air temperature (T_A). Pups were placed in the chamber on a raised platform constructed of polyethylene mesh, a surface that is non-conductive and allows for the free passage of air through the chamber. A mesh wall surrounded the platform to prevent pups from

touching the chamber walls directly. Holes in the side of the chamber, as well as in its plastic lid, allowed for the connection of thermocouples. Forced humidified air (300ml/min) entered the bottom of the chamber, flowed past the pup, and exited through the lid. Thermocouple leads for measuring physiological and air temperatures were attached to a National Instruments data acquisition device (USB-9211A), which sampled once per second per channel. All hardware and timing was controlled through LabView 2009 software.

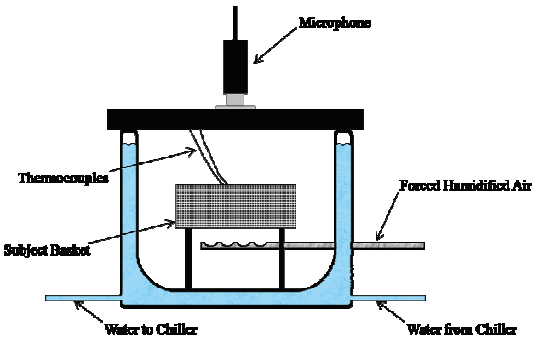

Figure 5: The thermogenesis testing apparatus. The subject is placed in the subject basket inside the double-walled glass chamber. The walls of the chamber are filled with temperature-controlled water, which actively regulates the internal temperature of the chamber. Forced humidified air is pumped into the chamber throughout testing. Subject and ambient temperatures were measured using thermocouples.

Chamber air temperature (T_A) and physiological temperatures were measured using Chromel-Constantan (T-Type) thermocouples. T_A within the metabolic chamber was measured using a thermocouple suspended 2 cm beneath the subject. Two physiological temperatures were acquired by attaching thermocouples just under the surface of the skin using callodion as an adhesive (Blumberg et al. 1996; Spiers et al. 1986). One thermocouple was attached in the interscapular region above the brown fat pad, thus providing a measure of interscapular temperature (T_{IS}) and BAT thermogenesis. The other thermocouple was attached in the lumbar region, and measured the temperature of the back of the subject (T_{Back}), a non-heat-producing region. The difference between T_{IS} and T_{Back} (T_{IS} - T_{Back}) was used to confirm the presence and degree of brown adipose tissue thermogenesis (Blumberg et al. 1996; Hull et al. 1965).

Ultrasonic recording equipment included model CM16/CMPA40-5V microphones (Avisoft Bioacoustics; Berlin, Germany) connected to a desktop computer through a National Instruments instrumentation recorder (PCI-6132). Microphone voltage was sampled at a rate of 1 MS/s (1 million samples per second) at 14 bit, which allowed for high fidelity recording at frequencies well beyond 100kHz, more than double the expected fundamental frequency range of 40-50KHz. The

microphones have a flat frequency response across the anticipated frequency range. National Instruments software (LabView 2009) began acquisition of ultrasonic vocalizations at the session start and terminated at the session end as described below. Recordings were conducted within the thermoregulatory test chambers as described above.

Testing Procedure

On PND 3 and 5, dams and their litters were removed from their home cage and transported in a small incubator to the surgical procedure room. One pup of each sex showing a milk band was removed from the litter, weighed, and placed into the incubator. Each pup was then anesthetized with isoflurane (5% for induction, 2% for maintenance), thermocouples were implanted, and pups were promptly returned to the incubator. The entire surgical procedure required less than 5 minutes and subjects were maintained at thermoneutral temperatures throughout surgery using heat pads. After surgery, subjects were transported in the incubator to the test room and placed into the thermal chambers. Data collection began following 60 minutes of habituation to the chamber at thermoneutral temperatures and continued for 2 hours using a series of thermal challenges. Since rat pups have exhibit age-dependent thermogenic capacities, age-appropriate thermal challenges were used (Blumberg et al. 1996). On PND 3, after 60-min of habituation at 36.0ºC, the apparatus temperature was reduced to 32.0ºC (a moderate temperature challenge) for another 60-min, and then again reduced to 28.5ºC (an extreme temperature challenge) for a final 60-min. On PND 5, pups were treated in the same manner, except that the habituation temperature was held at 35.0ºC, the moderate temperature challenge was 28.0ºC, and

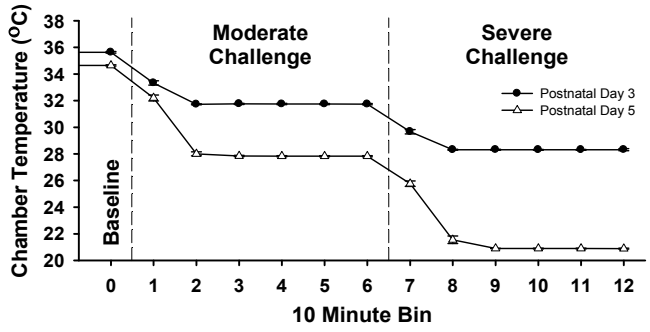

Figure 6: Environmental challenges posed on postnatal days (PND) 3 and 5. Subjects were first exposed to a baseline thermoneutral habituation period for one hour (PND 3: 36°C, PND 5: 35°C), then a moderate thermal challenge for one hour (PND 3: 32°C, PND 5: 28°C), and lastly an extreme thermal challenge for one hour (PND 3: 28.5°C, PND 5: 21°C).

the extreme temperature challenge was 21.0ºC. The actual thermal challenges used on both test days are presented in Figure 6. Biometric data were collected and vocalizations recorded continuously during the tests. Following testing, pups were returned to their litters. To ensure the same pups were not retested, they were marked with paw tattoos.

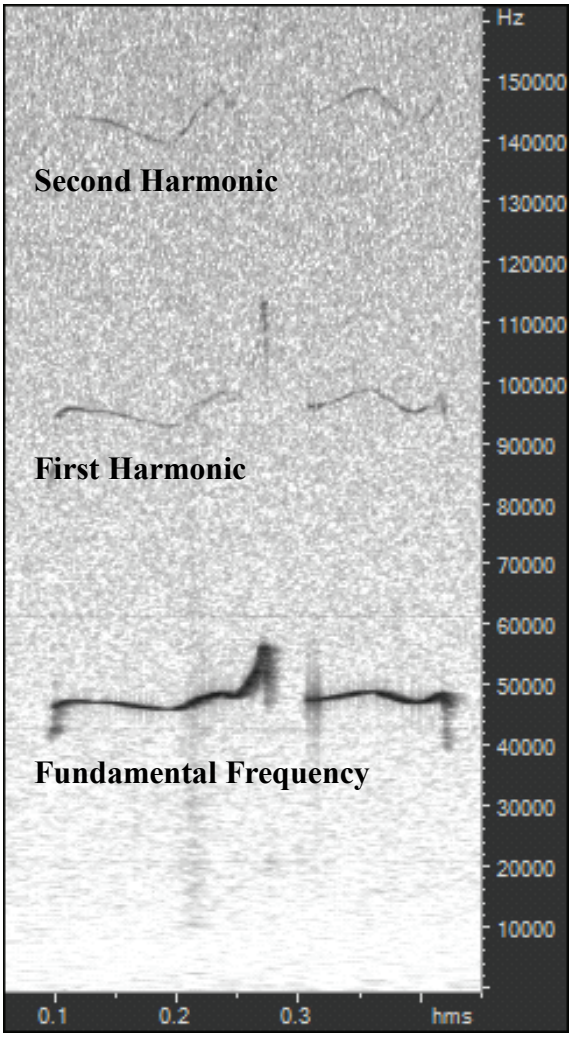

Figure 7: Explanation of acoustic measures of two pup ultrasonic vocalizations. The amplitude is denoted by the intensity at each point in the call.

Vocalization Measures

Ultrasonic vocalization analyses addressed likelihood to call, latency to first call from the start of the recording period, repetition rate**,** duration of call, and acoustic spectral differences. Acoustic properties included measures of pitch (highest frequency achieved by the fundamental component) and minimum frequency, measures of acoustic power such as the maximum amplitude (loudness) and number of harmonics visible (additional waveforms visible at multiples of the fundamental frequency), and the variation in amplitude and frequency of each call. These measures are detailed in Figure 7.

Data Analysis

Gestational data and subject body weight were examined using Analysis of Variance (ANOVA), followed by posthoc Tukey tests where appropriate. T_{IS}, T_{Back}, and T_{IS} - T_{Back} data were binned into 10-min intervals for both statistical and presentation purposes, and compared between

treatment groups and sexes using repeated measures general linear models, followed by posthoc Tukey tests where appropriate. No statistical comparisons between PND 3 and 5 could be made, because of the different age-appropriate thermal challenges used for each time point. To reduce to total number of comparisons, the focus of our analysis was on temperatures from the two thermal challenges. Data from the first 50 minutes of unchallenged thermal habituation were excluded from our statistical models. Data from the last 10-min bin of the habituation period was included in figures. Means and standard deviations are presented in figures.

Ultrasonic vocalization data was analyzed using generalized estimating equations, specifically Poisson regression. Data were adjusted for sex, group, body temperature (back or interscapular), and body weight to evaluate sex, group or sex x group differences for the number of calls within each PND (3 or 5). Further analyses included logistic regression to evaluate sex, group, or sex x group differences in the proportion of pups with/without calls. Frequency data were dichotomized into those vocalizations with frequencies above and below 75 kHz, based on pilot study results indicating that calls typically occur with fundamental frequencies of approximately 40-50 kHz or 85-95 kHz. To simplify the explanation of results, calls produced with fundamental frequencies below 75 kHz will be referred to as ‘low-pitch’ and those greater than or equal to 75 kHz will be referred to as ‘high-pitch.’

Results

Gestational Effects

Gestational data are detailed in Table 1. There were statistically significant differences in gestational weight gain of the dam [$F(2,54)=6.28$, $p\leq0.01$], with cocaine-treated dams gaining less weight over gestation than untreated dams ($p\leq0.01$); however, cocaine-treated dams gained more weight over PPDs 1-5 [$F(2,45)=8.49$, $p\leq0.01$] than untreated dams ($p\leq0.01$). Mean PND1 cocaine-exposed culled litter weights were also lower [$F(2,45)=7.57$, $p\leq0.01$] than both untreated ($p\leq0.01$) and saline-treated litters ($p\leq0.05$), reflected in individual pup weight differences rather than litter size at birth [$F(2,45)=5.52$, $p\leq0.01$] and on PND 3 [$F(2,48)=4.16$, $p\leq0.01$]. Thus cocaine-exposed pups that were tested had lower pup body weights than untreated pups on both PND1 ($p\leq0.01$) and PND 3 ($p\leq0.02$), but they were not statistically different from saline-treated pups on any day, and did not differ from either control group on PND 5. There were no statistically significant differences in gestational length, total number of pups in the litter, male to female pup ratio, or individual pup weights on PND 5.

Table 1: Gestational and Postpartum Data

Treatment Group	Dam Gestational Weight Gain (g)	Dam Postpartum Weight Gain (g)	Culled Litter Weight (g)	PND 1 Pup Weight (g)	PND 3 Pup Weight (g)	PND 5 Pup Weight (g)
Cocaine	123.5 ± 3.5 ^{U}	15.5 ± 2.7 ^{U}	60.3 ± 1.7 ^{U,s}	6.2 ± 0.2 ^{U}	7.3 ± 0.3 ^{u}	9.6 ± 0.4
Saline	139.5 ± 6.2	9.0 ± 3.3	65.6 ± 1.2	6.6 ± 0.1	7.5 ± 0.1	9.4 ± 0.3
Untreated	146.5 ± 4.2	1.4 ± 1.9	67.7 ± 1.3	6.8 ± 0.1	8.0 ± 0.1	10.1 ± 0.2

Note: Superscripts indicate statistically significant difference from respective group (U: untreated, S: saline). Uppercase superscripts indicate significance of $p\leq0.01$, lowercase superscripts indicate $p\leq0.05$.

Thermoregulation-Specific Effects

Figure 8 indicates that on PND 3, there was a significant main effect of treatment on interscapular [$F(2,83)=12.01$, $p\leq0.01$] and back temperatures [$F(2,82)=15.63$, $p\leq0.01$], such that both cocaine- ($p\leq0.01$) and saline-exposed pups ($p\leq0.01$) were significantly warmer than untreated pups in these two regions. Additionally, the interaction between treatment and time was also significant for both interscapular [$F(24,978)=3.10$, $p\leq0.01$; Figure 8a] and back temperatures [$F(24,957)=2.41$,

p≤0.01; Figure 8b] in those groups. As time progressed and environmental temperatures decreased, the higher interscapular temperatures of both cocaine (bins 5-12) and saline-exposed pups (bins 3-12) relative to untreated animals increased even further (significance ranged from p≤0.05 - p≤0.01). This effect was also evident in the back temperatures of cocaine-exposed (baseline and bins 1-12, p≤0.05) and saline-exposed pups (baseline and bins 1-12, p≤0.05). Despite these differences, there were no significant differences between groups on T_{IS} - T_{Back} data (see Figure 8c), implying that cocaine and saline-exposed pups were not generating significantly different amounts of heat from BAT than untreated pups. There were also no sex differences in any measure (data not presented).

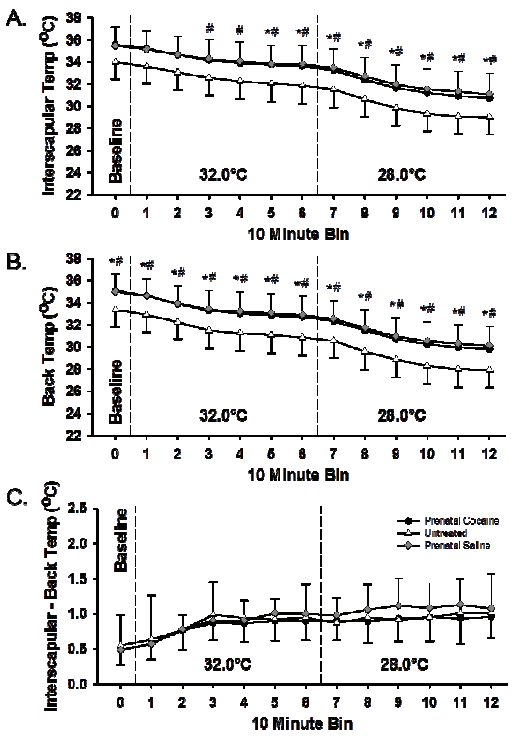

Figure 8: Postnatal day 3 pup thermoregulation and thermogenesis. Cocaine and saline-exposed pups demonstrated significantly higher interscapular (A) and back (B) temperatures than untreated pups during the specific 10 minute bins denoted (cocaine: *, saline: #), but showed no alterations in the difference between these measures (C), indicating that they did not differ in their thermogenic capabilities.

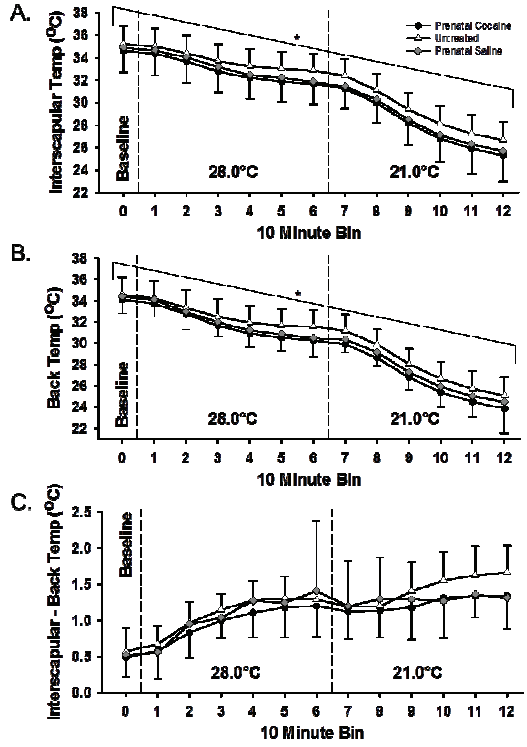

Figure 9: Postnatal day 5 pup thermoregulation and thermogenesis. As indicated by asterisks, cocaine-exposed pups demonstrated significantly lower interscapular (A) and back (B) temperatures than untreated pups across the testing period, but did not differ from saline, and showed no alterations in the difference between these measures (C), indicating that they did not differ in their thermogenic capabilities.

As shown in Figure 9, on PND 5 there was a significant main effect of treatment on both T_{IS} [$F(2,88)=3.66$, $p\leq0.03$; Figure 9a] and T_{Back} [$F(2,85)=3.25$, $p\leq0.04$; Figure 9b]. Throughout the thermal challenges, cocaine-exposed pups demonstrated generally lower T_{IS} and T_{Back} temperatures than untreated pups ($p\leq0.05$). However, there was no significant interaction between temperature and time. Additionally, as for PND 3 subjects, there were no significant differences in T_{IS} - T_{Back} (see Figure 9c). Sex was also not a significant factor in any temperature differences during the thermal challenges (data not presented).

Ultrasonic Vocalization-Specific Effects

The vocalization results were highly dependent upon the thermal state of the pup, indicating that at colder back temperatures, pups were more likely to vocalize on both PND 3 ($p\leq0.05$) and PND 5 ($p\leq0.01$) regardless of exposure condition or sex. Additionally, the maximum amplitude of the call also increased as pup back temperature decreased on PND 5 ($p\leq0.01$). On this day the effect of pup temperature on the peak frequency differed by the pitch of the call, in that high-pitch calls decreased in frequency as temperature decreased ($p\leq0.01$), but low-pitch calls increased in frequency as temperature declined ($p\leq0.01$). This effect was mirrored in the minimum frequency of the call (high-pitch, $p\leq0.01$; low-pitch, $p\leq0.01$); however, regardless of temperature, the ratio of the number of low-pitch to high-pitch calls did not change significantly. Back temperature was also strongly associated with changes in frequency variation on PND 5, but not with changes in amplitude variation. At lower temperatures, more variation in frequency was found in each call ($p\leq0.01$). Changes in back temperature were not associated with any change in the number of harmonics on either PND 3 or 5.

As shown in Figure 10, after adjusting for the effect of back temperature on both test days, pups that weighed more were more likely to produce a call and their calls were greater in amplitude (PND 3, $p\leq0.01$; PND 5, $p\leq0.01$). Pups that weighed more were less likely to produce high-pitch calls on PND 3 ($p\leq0.01$), and on both test days, the peak and minimum frequencies of high- (PND 3, $p\leq0.01$; PND 5 $p\leq0.01$) and low-pitch calls (PND 3, $p\leq0.01$; PND 5 $p\leq0.01$) were lower. While no

effects of weight on the number of harmonics, frequency variation, or amplitude variation were found on PND 5, heavier pups were found to produce fewer harmonics on PND 3 ($p \leq 0.01$), as well as have more variation in frequency and amplitude within each call ($p \leq 0.01$).

Despite these relationships between body weight and vocalizing, females were still more likely to produce calls on PND 5 ($p \leq 0.01$), but not on PND 3. However, on PND 3, the low-pitch calls produced by females were typically of a lower peak frequency ($p \leq 0.01$) and minimum frequency ($p \leq 0.01$) than the low-pitch calls produced by males. The sex of the pup was not associated with any noteworthy differences in frequency or amplitude on PND 5.

There were no differences in the number of calls produced by untreated, cocaine- or saline-exposed pups on PND 3 or 5, although variability in this measure was extremely high on both days. On PND 3 there was a significant difference in the proportion of pups in each group that called at all. Pups from the untreated group were four times more likely to have at least one call than pups from the cocaine-exposed group ($p \leq 0.01$; see Figure 10) and five times more likely than saline-exposed pups ($p \leq 0.05$). There were no significant differences in the likelihood to call between cocaine- and saline-exposed pups. In addition to calling more on PND 3, untreated pups were also significantly more likely produce high-pitch calls than the saline-exposed pups ($p \leq 0.01$), and non-significantly more than cocaine-exposed pups ($p \leq 0.09$). Despite these differences in the likelihood to call, there were no significant differences in the peak frequency, minimum frequency, amplitude, number of harmonics, or frequency/amplitude variation between the treatment groups.

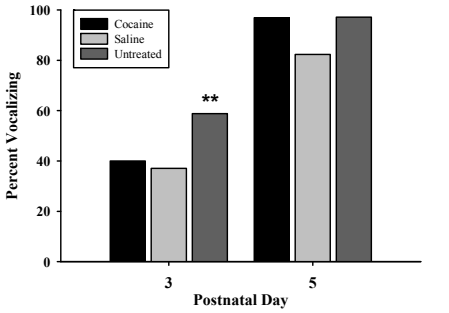

Figure 10. Percent of pups that vocalized on PND 3 and 5.

Discussion

The data presented here in part supported our hypotheses that prenatal exposure to cocaine would alter measures of early postpartum thermoregulatory ability vocalizing behavior and that it does so similarly in both male and female rat pups, although the group effects differed between PND 3 and 5. The fact that chronic saline exposure was also associated with altered thermoregulation and vocalization was not predicted and indicates that differences in cocaine-exposed pups are likely not simply a result of drug exposure itself. Whether these effects were directly related to prenatal cocaine exposure or through the secondary effects of prenatal cocaine, such as prenatal stress, cannot be determined from the current dataset, although the similarities between the cocaine- and saline-exposed animals would suggest either similar mechanisms or concurrently occurring, but different mechanisms of action in both groups. Barry Lester and colleagues (Lester et al. 2009) proposed three primary avenues by which fetal cocaine exposure may alter development: direct neurotransmitter modulation, vasoconstriction, and fetal programming. While fetal cocaine and saline exposure would not likely result in similar neurotransmitter modulation, it is possible that maternal stress caused by early food restriction and repeated saline injections could increase plasma concentration of catecholamines in both groups of dam (saline and cocaine-treated), reducing placental blood flow (Jansson 1988), and thus reduce fetal oxygen and nutrient supply, potentially causing hypoxia in the developing fetus. Catecholamine levels could then also be elevated in the developing fetus, resulting in fetal vasoconstriction and potentially resulting in further hypoxia in the developing brain (Jensen *et al.* 1987). Such effects may impact both cocaine and saline-exposed groups to different extents, as effects were not as severe in saline-exposed animals.

Prenatal malnutrition is potentially a secondary contributing factor, as maternal cocaine use has been strongly associated with malnourishment of the mother and fetus and placental vasoconstriction further complicates nourishment delivery to the fetus. As is the case with fetal cocaine exposure, under- or over-provision of nutrients and postnatal nutritional challenges can alter the long-term adipose tissue volume and function (Mostyn et al. 2009), potentially leading to obesity,

diabetes, and disruptions in renal function (Cinti 2006). Unfortunately, no measures of diet or nutrition were examined in these animals, thus the contribution of this mechanism cannot be determined from the data presented here, aside from the measures of litter and individual pup weight gain; however, prenatal malnutrition alone has been shown to have no effect on early life dam-pup interactions (Tonkiss *et al.* 1995). Saline-treated control dams were employed in addition to the untreated controls as a specific control for prenatal stress, both from repeated daily injections and for cocaine-induced anorexia as mentioned previously. Prior studies have reported both significant and non-significant differences between untreated and saline-treated controls concerning both mothers and offspring, and stress effects are often considered to be an aspect of cocaine's effects in animal studies. Our modified pair-feeding paradigm was designed to offset some concerns regarding continued food deprivation; however, some effects, though limited, are likely. The long term effects of cocaine and saline exposure likely differ despite the possible common mechanisms, and the interactive relationship between stress, malnourishment, and the specific effects of fetal cocaine exposure is currently unknown.

With respect to prenatal cocaine exposure, body temperature is a relatively novel developmental target, but the presence of such effects are not necessarily surprising given the disturbances in cardiac development seen in similarly exposed pups (Regalado *et al.* 1996; Sun *et al.* 2003) and prior reports of acute cocaine altering thermoregulation in adults (Crandall et al. 2002). Additionally, the magnitude of group effects was considerably stronger than we had anticipated; especially on PND 3. At this time point, the externally apparent temperature of cocaine- and saline-exposed pups was almost two degrees (Celsius) warmer than untreated pups on average; a difference that was maintained despite alterations in environmental temperatures. Hyperthermia of only two degrees Celsius would not necessarily constitute a fever, but long-term elevation of body temperature could have significant developmental impacts, which again may be further exacerbated by prenatal cocaine exposure. Infant hyperthermia alone has been shown to intensify drug-induced neurotoxicity (Crandall et al. 2002), worsen hypoxia-induced brain damage (Kiyatkin 2007), dramatically alter

neurotransmitter levels (Laptook et al. 2002), and may alter brain development in numerous regions already be targeted directly by cocaine (Cremades et al. 1982). Infant hyperthermia has even been suggested as a potential contributor to Sudden Infant Death Syndrome (Baumgart 2008), which is more likely to occur in cocaine-exposed infants (Kinney 2009). Thus, despite the similarity in results between the cocaine and saline groups, the developmental repercussions of such effects may differ between these two groups.

In spite of the increase in temperature in cocaine-exposed pups on PND 3, a decrease in temperature was seen only two days later in this group, on PND 5. This shift in body temperature relative to controls was unanticipated and highly interesting. There were no significant differences in the amount of heat generated by any of our pup groups at either testing time point (PND 3 or 5), suggesting that any differences in apparent body temperature were likely the result of alterations in heat distribution within the body (the heat produced in the interscapular region was more quickly dissipated to non-heat producing regions). Such effects would suggest non-metabolic mechanisms, and potentially point to cardiac or circulatory system effects. Brown adipose tissue primarily surrounds the heart and is also distributed throughout the peritoneum. Its primary location proximal to the heart is of particular importance, as the heart acts as a pumping mechanism to distribute the heat generated by BAT throughout the body via the circulatory system. Considering how strongly brown adipose tissue thermogenesis is tied to cardiac rate (Blumberg *et al.* 1997; Sokoloff *et al.* 1998), our lack of groups differences in T_{IS}-T_B (our measure of BAT thermogenesis) would suggest that cardiac rate may not be the primary mechanism. Instead, cardiac stroke volume, blood pressure, or other circulatory system characteristics may be more significant contributors.

In an attempt to address this question, we have examined heart volume and beta-adrenergic receptor levels in cardiac tissue collected from a separate group of animals. This study (unpublished) found no change in beta-adrenergic receptor levels following prenatal cocaine exposure, but did show reductions in cardiac mass (see Figure 11 below), which should be directly proportionate to cardiac volume. Such findings would suggest that differences in thermoregulation following prenatal cocaine

or saline may be resultant from altered cardiac stroke volume as opposed to the contractile mechanisms. However, without cardiac data from saline-exposed pups, it is difficult to tell if these effects are specific to cocaine.

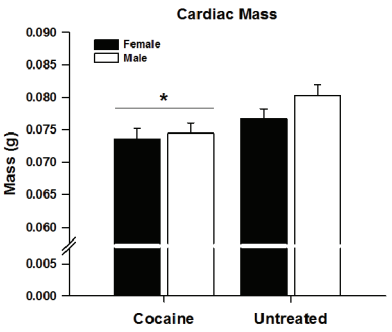

Figure 11. Cardiac mass on postnatal day 5. Cocaine exposed males and females show reduction in cardiac mass compared to untreated pups.

While altering steady-state body temperature likely has implications on the development of other physiological systems, such effects would also likely be detectable by the effected pup's mother, influencing her behavior. A feedback system exists between the pup and mother, such that maternal heat is transferred to pups during close contact, but pups also act as a source of heat for mothers (Woodside et al. 1988). Thus, not only does a mother have incentive to isolate hyperthermic pups to reduce the body temperature of the pups, but also to reduce her own body temperature. Abnormally warm pups, such as the PND 3 cocaine-exposed pups seen in the current study, would offset this feedback loop in favor of reduced maternal attention and increased isolation. Indeed, cocaine-exposed pups have been shown to receive less direct contact from dams, regardless of dam drug exposure (Johns *et al.* 2005a), especially very early in the postpartum period (days one through three). Later in the postpartum period, maternal care differences are less apparent, just as the temperature differences between the groups presented here were less severe. The inverse relationship between these two variables (heat and maternal attention) seems to fit nicely with the thermal data reported here on PND 3, and may present a potential factor in the patterns of maternal care deficits reported earlier (Johns *et al.* 2005a), although this was not directly tested in the present study. Follow-up studies of this system should include the external control of pup body temperature to assess direct effects on maternal contact.

While the thermal state of pups alone can influence maternal attention, the thermal state of the pup also contributes to the production of ultrasonic vocalizations in the early postpartum, another cue important for the elicitation of care. One physiological mechanism of cry production, laryngeal braking, has been suggested as a potential link between these two behavioral systems (Blumberg et al.

2001). As a pup becomes colder, it is more likely to use this technique to boost metabolic and cardiac output, thus briefly warming the pup, but also producing a vocalization as a byproduct. The strong relationships between both the weight of the pup and the temperature of the pup and its likelihood of vocalizing found here strongly support this mechanism of vocalization production; however, it is unknown how the thermal state of the pup contributes to the frequency, amplitude, etc.

Aside from physiological mechanisms, older pups with stress systems intact may also be experiencing stress related to the thermal challenge itself or resulting from isolation from littermates and maternal attention. In addition to vocalization production, a number of other attributes were found to be altered by the thermal state of the pup, such as the frequency, amplitude, and the variation in frequency of the call. While it is possible that such elements are the result of changes in the physiological mechanisms of cry production, it is just as likely that such elements reflect the psychological state of the pup. Such differences in mechanism might be reflected in the differences in these factors between PND 3 and 5. It is unknown exactly when stress systems become active, but in older animals with intact stress systems, applying psychological stress often results in vocalization production (Sanchez 2003). Holding animals at thermoneutral temperatures and applying non-thermal stimuli to elicit vocalizations (eg pain) may shed light on this question.

The data we present here are robust and interesting; however, they must be interpreted with caution. Considering the deficits in maternal attention reported for cocaine-treated pups in the early postpartum, period, without using a cross-fostered group it is difficult to separate the respective contributions of prenatal cocaine and maternal cocaine treatment. Further mechanistic studies of other pup characteristics, as well as cross-fostering studies, could address these points, since the cues examined here are only one of many cues that are relevant to care. It is probable that thermal state can also influence other pup behaviors, interacting with other cues to effectively elicit care. Additionally, given the similar direction and magnitude of cocaine and saline exposure effects on PND 3, it will be important to determine differences in stress-related effects aside from those attributable to cocaine treatment alone, and how these effects interact to further alter development.

CHAPTER IV

A Study of Prenatal Cocaine's Effects on Urine Constituents

Introduction

In addition to the auditory and thermal cues discussed in Chapter III, olfactory cues are important to a dam's ability to locate pups (Farrell et al. 2002a; Farrell et al. 2002b) and are thought to play a direct role in the initiation of maternal behavior (Morgan *et al.* 1992). It has been hypothesized that pup odors activate olfactory nuclei that exert an inhibitory effect on brain regions implicated in maternal behavior (Fleming et al. 1974a; Fleming et al. 1974b). Lesioning the main olfactory bulb will not eliminate maternal care, but does increase the latency to retrieve pups (Benuck et al. 1975; Fleming et al. 1974a; Kolunie et al. 1994). Disruption of olfactory bulb function may have many downstream effects of behavior given its direct neuronal connections to the supraoptic nucleus of the hypothalamus and amygdala (Yang *et al.* 1995).

Pup-produced olfactory cues are mainly attributed to excretions, either from urine, feces, or the preputial gland, released by maternal licking in the early neonatal period (Capek et al. 1956; Friedman et al. 1981; Gubernick et al. 1983). Dams lick pups, thereby ingesting urine after delivery, to stimulate urination and defecation in the pups (Alberts et al. 1990) and to rehydrate themselves (Friedman et al. 1981; Gubernick et al. 1983). Olfactory cues influence maternal behavior in many mammalian species (Levy *et al.* 2004); even human mothers can detect subtle olfactory differences in babies (Bonnin *et al.* 1990). Rat dams prefer odors associated with pups or other rat dams, and disruption of the olfactory system can affect maternal retrieval (Bauer 1983; Bauer 1993; Kinsley et al. 1995; Magnusson et al. 1995; Malenfant et al. 1991). These results imply a difference in the

reinforcing properties of familiar versus unfamiliar pup cues (Lee et al. 2000; Mattson et al. 2001; Mattson et al. 2003; Mattson et al. 2005).

To date, very little research has examined the particular compounds in pup urine that may be detected by rodent mothers. However, one compound, dodecyl propionate (DP), has garnered particular interest. DP is secreted by the rat pup preputial gland, and appears to regulate rather than stimulate the amount and quality of anogenital licking that a pup receives (Brouette-lahlou *et al.* 1991; Brouette-Lahlou *et al.* 1991). While pups with ablated preputial glands still received small amounts of licking, they also had dramatically increased mortality rates related to significant reductions in anogenital licking compared to sham controls. It has been suggested that DP, in addition to the level of testosterone in pup urine, may in part explain while males pups are typically licked more frequently than female pups (Brouette-Lahlou *et al.* 1991).

Aside from its utility as a maternal cue, pup urine can also provide valuable information regarding the general health of the pup in the early postpartum period. Urinalysis in humans has been used for decades as a screening and diagnostic tool, because it can detect substances or cellular material in the urine that are associated with many metabolic, kidney, and urinary tract disorders. Additionally, urine can also provide a non-invasive means of detecting drug presence, such as cocaine and its metabolites. Cocaine is typically eliminated from plasma by hydrolysis, and about 75-90% is eliminated in the urine as either ecgonine methyl ester or benzoylecgonine (Ambre 1985). Although drug clearance rates are fairly well understood in adult rats, the clearance of cocaine in young pups is poorly understood. Additionally, prenatal cocaine exposure likely alters drug metabolism and may alter pharmacokinetics through its impact on liver and renal function. Prenatal cocaine's teratological effects on the renal system have been well documented in clinical research, with such infants showing a four-fold increase in the likelihood of urinary tract infections (Gottbrath-Flaherty *et al.* 1995), renal hypertension (Ho *et al.* 1994), and elevated bilirubin levels indicative of liver dysfunction (Wennberg *et al.* 1994). To our knowledge, no study of prenatal cocaine's effect on the developing renal or

urinary systems have been conducted in animal models, but given the effects reported in humans, such findings are likely.

The purpose of the current study was to determine the level of cocaine present in urine collected from young pups following prenatal cocaine exposure, and to examine levels of DP to determine if this may play a role in prenatal cocaine's effect on pup elicitation of care. We hypothesized that cocaine and its metabolites would be apparent immediately following birth, but will not persist past PND 2 given previously reported clearance rates in adults. We also hypothesized that DP levels will be lower in cocaine-exposed pups, in line with the reduced licking these pups have been reported to receive (Johns *et al.* 2005a).

Methods

Breeding

Individually housed 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 one week and then mated until conception was noted by the presence of a vaginal plug and sperm in a vaginal smear (gestation day (GD) 0). Following conception, females were randomly assigned to chronic cocaine, chronic saline, or untreated groups as they became pregnant (see below for treatment information). Weight gain was measured daily for all animals throughout gestation. Water and chow were available ad libitum for all except chronic saline-treated rat dams, who were matched with a chronic cocaine dam on a pair-feeding schedule to control for any effects of cocaine-induced anorexia. Seven days following conception (GD 7), females were moved to a colony room and individually housed on a regular 12:12 light:dark cycle with lights on at 7:00 AM. This procedure results in the majority of dams delivering in the normal daylight hours (Mayer et al. 1998). PPD 1 was defined as the calendar day during which delivery was completed. Following delivery, litters were culled to 10 pups (5 male, 5 female) and pups were returned to their own biological mothers. We chose not to employ surrogate mothers to control for differences in postnatal care between groups as we wished to study the more natural interactions of mothers and pups, as occurs in human clinical populations.

Dam Treatment

Females were randomly assigned to chronic cocaine, chronic saline, or untreated groups as they became pregnant, with 15 animals per group. Chronic cocaine-treated dams received twice-daily subcutaneous injections of 15 mg/kg of cocaine hydrochloride (total 30 mg/kg dose calculated as free base, 2 ml total volume, Sigma, St. Louis, MO) dissolved in normal saline at approximately 9:00 AM and 4:00 PM throughout gestation (GD 1-20) and not thereafter. This is the lowest chronic gestational cocaine treatment dose consistently reported to produce significant effects on pup-directed maternal behavior (Nelson et al., 1996). To prevent skin lesions, injections were alternated daily between rear

leg flanks. If a lesion appeared, the fur was clipped at the site, cleaned daily with a betadine solution, and a topical antibacterial ointment (Polymycin-Bacitracin-Neomycin, E. Fougera & Co., Melville, NY) was applied to the area. These measures have been shown to minimize skin lesion appearance and severity and have been used in numerous studies (McMurray *et al.* 2008b).

Control groups included both chronic saline-treated and untreated dams. Chronic saline-treated dams received injections of normal (0.9%) saline (2ml/kg total volume) on the same schedule and regimen as the chronic cocaine dams. Saline-treated dams were pair-fed to chronic cocaine dams in the early gestation period such that the amount of food ingested by cocaine-treated dams on average on a specific gestation day during the first 7 days of gestation was the amount provided to saline-control dams on the corresponding gestational day. Beginning GD 8, saline-treated dams had free access to rat chow. This procedure accounts for the time when cocaine-treated dams are most affected by the anorectic effects of cocaine in prior studies (Johns *et al.* 2005a; McMurray *et al.* 2008b), while not inducing the serious confound of food deprivation in saline-treated dams. Untreated control dams received no drug treatment or food restriction during gestation or during the postpartum period, but were weighed daily to control for the effects of handling.

Urine Collection and Analysis

Urine was collected in a pipette from all pups in test litters on PNDs one and three after gently stroking the pup's genital region with a soft paintbrush to elicit urination. Urine from pups of the same sex within each litter was pooled to aggregate a minimum of 50 µl total. After collection, urine was rapidly frozen on dry ice and stored at -80°C. This method has worked well in preliminary studies where urine was analyzed with a dipstick test (Siemens MultiStix 10SG). Urine analysis was completed by the Center for Human Toxicology at the University of Utah and the Biomarkers Facility Core at the UNC Gillings School of Public Health, Center for Environmental Health and Susceptibility. Samples were transported on dry ice to each research facility for analysis. Samples were analyzed for the presence and quantity of cocaine and metabolites (benzoylecgonine and

ecgonine methyl ester) using liquid chromatography in combination with mass spectroscopy (LC/MS), specifically electrospray ionization-mass spectroscopy, as previously reported (Lin *et al.* 2001; Lin *et al.* 2003). Detection of DP and level quantification was conducted via liquid chromatography followed by atmospheric pressure chemical ionization-mass spectroscopy. Since no published methods exist for the quantification of this chemical using these techniques, a series of studies took place to develop these methods for use in house. Additionally, since no standards were commercially available, standards first had to be synthesized in house. This was also completed by the Biomarkers Facility Core and validated using commercially available samples of butyl laurate, a chemically similar compound. LC/MS detection of the standards can be seen in Figure 12. The mass of DP was determined to be 243 m/z and the product mass 75 m/z. Once standards were synthesized and validated, detection of DP occurred via a series of studies. First detection was conducted in a non-concentrated 10 µl sample of urine, then in a non-concentrated 10 µl sample spiked with 1.4 nmol DP standard. Next, urine protein was ethanol-precipitated to reduce background noise, and both non-spiked and spiked samples examined. Following this, urine samples were chloroform-extracted to further optimize the procedure, and again, spiked and non-spiked 10 µl samples were examined.

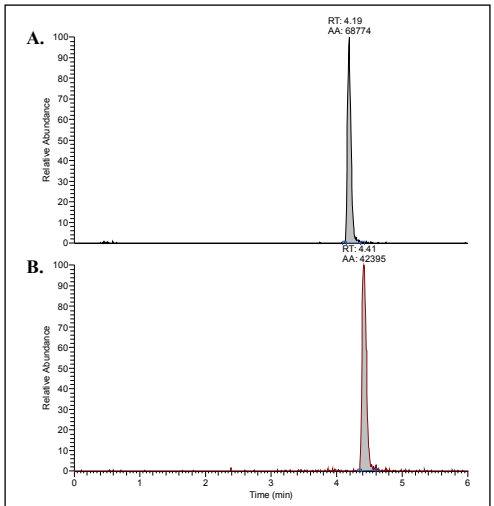

Figure 12. LC/MS results from standards: (A) dodecyl propionate and (B) butyl laurate.

Data Analysis

Individual LC/MS data points were collected by the associated software package. Collected data was then compared between treatment groups, sexes, and across test days using ANOVA, with alpha levels of 0.05, and Tukey posthoc tests to examine specific effects.

Results

On both PND 1 and 3, cocaine and its primary metabolites (benzoylecgonine and ecgonine methyl ester) were detected in both male and female cocaine exposed pup urine samples (see Figure 13), but not in untreated pups (data not shown). Considerably lower levels were detected on PND 3 compared to PND 1. There were no differences between males and females on either day.

No DP could be detected above background in either processed or unprocessed urine samples using the methods reported above. DP was detected only in the samples that had been spiked with 1.4 nmol standard, suggesting that if DP is present in pup urine, it is at concentrations below the lower limits of quantification with LC/MS. Example data is shown in Figure 14.

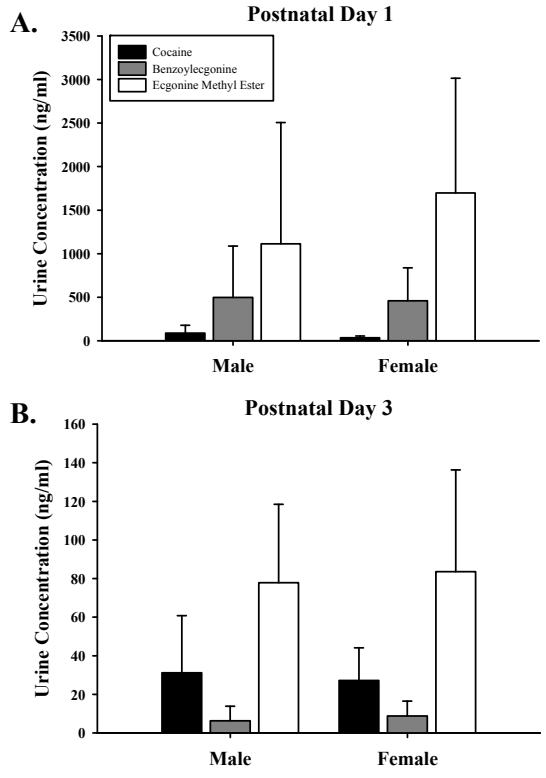

Figure 13. Levels of cocaine and metabolites in pup urine following prenatal cocaine exposure on PND 1 (A) and PND 3 (B).

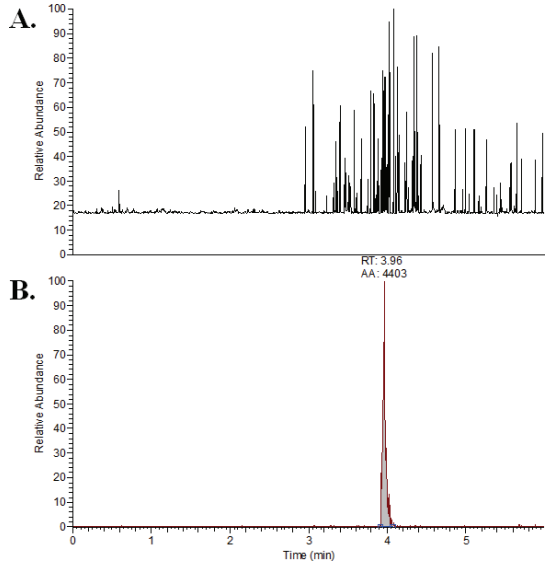

Figure 14. Detection of dodecyl propionate in unprocessed pup urine (A) and urine spiked with 1.4 nmol dodecyl propionate (B). No dodecyl propionate was seen above background in A.

Discussion

The purpose of this study was to determine if cocaine and its metabolites were present over the 5 days following birth in the urine of pups exposed to cocaine in utero, and to determine if levels of DP in pup urine were reduced following prenatal cocaine exposure. Although we were able to demonstrate that both cocaine and its metabolites are present in pup urine up to 3 days postpartum, we were unable to successfully isolate DP in any urine sample. It is surprising that cocaine is still present in pup urine on PND 3, at least 24 hours after the last cocaine treatment was administered to their mother on gestation day 20. One study using similar methods found that cocaine plasma levels in a directly treated rat peak in 1-2 hours and remain elevated for up to 5 hours following a 40 mg/kg subcutaneous injection of cocaine (Vernotica et al. 1998), with urinary cocaine levels following similar timelines. While this dosage is slightly higher than the one used here, similar rates of clearance were expected from our dosage despite the indirect means by which cocaine was administered to the developing pup. The extended duration of cocaine's presence found here could be related to either reduced metabolism of cocaine by the pup or reduced excretion of waste, possibly from less maternal licking, allowing levels to accumulate in the bladder. Considering the undeveloped state of pup liver and kidneys, it is likely that cocaine would persist for longer durations in plasma, altering the teratological time course and potentially influencing maternal care by altering the gustatory or olfactory properties of pup urine when finally excreted. To date no studies have been published on the reward value of cocaine containing urine compared to non-cocaine containing urine in rodent mothers, although such studies are underway at this time.

The pharmacokinetics of prenatal drug exposure are poorly understood in general, and considering the numerous methods in which cocaine has been administered in prior studies (inhalation, intravenous, intraperitoneal, etc.) and the varying durations of exposure, the conclusions from the pharmacokinetic studies that have been completed are highly variable. For example, fetal plasma half-life has been reported in humans to range from as few as 4 minutes (Burchfield *et al.*

1990) to as many as 55 minutes (Robinson *et al.* 1994) depending on the route used and the number of infusions given. Despite this variability, it is generally agreed upon that cocaine is metabolized via first-order pharmacokinetic processes in the fetus as well as the mother (Downs *et al.* 1996), thus clearance of the drug from plasma would depend solely upon the dose of drug given.

Estimates of the concentration of cocaine in the brain and plasma were approximately 2-3-fold less in fetuses than in their dams (Spear *et al.* 1989), highlighting the role that the placenta plays in restricting cocaine's entry into fetal circulation. Despite the differing levels, the brain/plasma cocaine ratio was equivalent in both parties. Thus, once past the placental barrier, cocaine appears to have equal affinity for brain tissue. Like cocaine, very little (approximately 2%) benzoylecgonine in maternal blood crosses the placenta in sheep (Covert *et al.* 1994), suggesting that the primary source of benzoylecgonine in the developing fetus is from the metabolism of cocaine within the fetus itself. However, in rats, benzoylecgonine can actually be found in greater concentrations in fetal brain than in maternal brain (Spear *et al.* 1989). This is particularly troubling, considering benzoylecgonine has been shown to cause vasoconstriction of cerebral arteries (Dixon et al. 1989; Madden et al. 1990; Schreiber et al. 1994). Such effects may explain in part the increased risk hypoxic-ischemic injury in infants with prenatal cocaine exposure. Benzoylecgonine levels have also been associated with alterations in infant behaviors. Neonates with increased signs of "neuroexcitation" had benzoylecgonine but no cocaine in urine, whereas "lethargic" neonates had detectable levels of urinary cocaine (Konkol *et al.* 1994). Considering such reports, the persistent elevation of urinary benzoylecgonine levels through PND 3 in our samples may have implications on fetal blood flow and brain development long after direct cocaine exposure has ceased. It would be most interesting to examine levels of these compounds in brain to determine how long pharmacologically relevant levels persist.

The second goal of this study was to examine levels of DP in pup urine following prenatal cocaine exposure. As mentioned in the introduction, this chemical is thought to reinforce maternal licking of pups (Brouette-lahlou *et al.* 1991; Brouette-Lahlou *et al.* 1991). Unfortunately, we were

unable to quantify its levels in pup urine using the methods reported here. This may be due to a number of factors, including the techniques used to collect and store the urine, and the methodology for sample analysis. As implied by its chemical structure, DP is relatively oily, and it is possible that it would stick to the skin of the pup when excreted from the preputial gland and remain separate from the urine. Urine samples were collected from our subjects by suctioning the urine as it was secreted, thus potentially not collecting DP had it remained apart from the pool of urine. Studies that have effectively isolated DP (Brouette-lahlou *et al.* 1991; Brouette-Lahlou *et al.* 1991) directly extracted it from the pup preputial gland, bypassing its relevance as a stimulus for licking by the dam. However, even if DP had been successfully collected using these methods, because of its oily nature it is equally likely that it would have separated from the urine in our collection vials prior to freezing. It then could have affixed to the side of the vial and potentially not been collected when the sample was transferred for LC/MS analysis; however, the ethanol and chloroform extraction techniques were used to minimize the potential for this confound.

Since LC/MS was clearly not effective in the current study, future investigations of DP should use other techniques for isolating the compound, such as radioimmunoassay or gas chromatography. These techniques traditionally have much greater signal to noise. Those studies reporting successful isolation of DP (Brouette-lahlou *et al.* 1991; Brouette-Lahlou *et al.* 1991) used gas chromatography followed by mass spectroscopy. We opted not to use such systems because of their lack of availability at the time of study design, and because we hoped to include our analysis of DP in a larger metabolomics-type study of pup urine, which would have required LC/MS. Unfortunately, this was not possible in the end, leaving our question about DP unanswered at this time.

CHAPTER V

GENERAL DISCUSSION

These studies were designed to investigate how cocaine exposure during the gestational period influences the interactions between mothers and offspring including possible mechanisms involved in group differences. The first experiment identified a variety of disruptions in postpartum maternal responses to infants as well as long-term intergenerational effects on maternal-social behavior attributable to maternal drug treatment, pup prenatal drug exposure, or the interaction of these two factors in the postpartum and postnatal periods. One of the most important findings from this study was that offspring with prenatal cocaine exposure were less able to elicit optimal care from any mothers, drug treated or otherwise, during the early postpartum period. Thus, the second experiment was designed to investigate how prenatal cocaine exposure may alter a number of specific attributes and behaviors of pups known to elicit normal maternal care. The results from this study indicated that prenatal cocaine exposure can result in differences in thermoregulatory and vocalizing behavior in pups, but that these effects weren't clearly dissociable from the possible effects of prenatal stress alone. These findings contribute not only to our understanding of cocaine's effects on mother-infant interactions, but also to our understanding of cocaine's effects on development in male and female offspring, a field of study that has suffered from a history of ill-conceived research and much political controversy. The final experiment was designed to determine the duration of cocaine and its metabolite's presence in pup urine following prenatal cocaine exposure, as a possible contributor to pup behavior and maternal response to olfactory or gustatory stimuli. This study found that cocaine was present in pup urine through at least postnatal day 3. This finding has implications

on questions surrounding the teratological effects of prenatal cocaine, as it is likely that cocaine may have a much slower clearance rate in pups than was previously understood.

This body of work derives from a common theoretical framework, concerning the impact of cocaine on mother-infant interactions. As detailed in Figure 15, this framework suggests that cocaine's effect on development is determined by 1) its indirect effects on the mother's maternal behavior; 2) its direct impact on developmental biology; and 3) by its indirect effects on pup stimulation of maternal care, mediated through its effects on developmental biology. This framework will be used to guide the discussion below, highlighting the interaction between the altered maternal environment and the altered postnatal development and behavior of the pup.

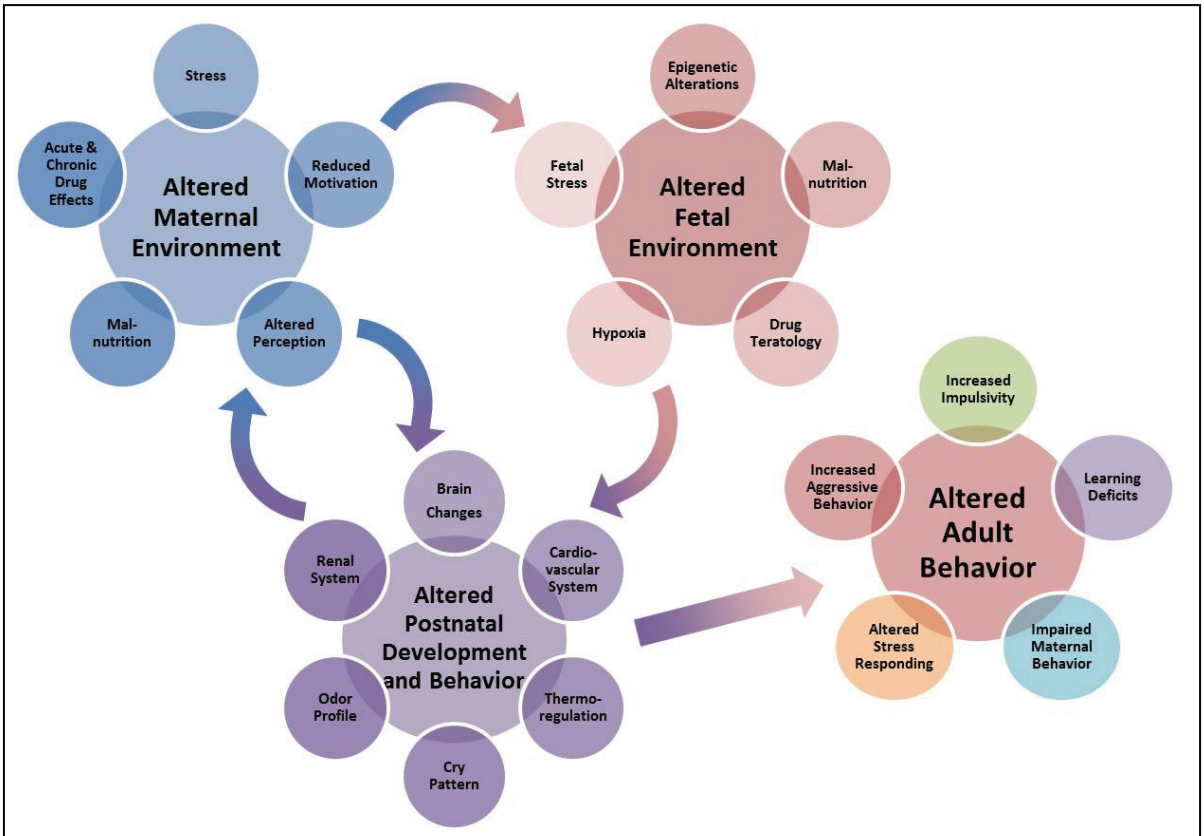

Figure 15. Theoretical diagram of the effects of maternal cocaine use on the next generation.

Alterations to the Maternal Environment

The effect of maternal cocaine treatment on maternal behavior has been well documented, both in Chapter 2 (Johns *et al.* 2005a) as well as in numerous other studies too extensive to detail (Johns et al. 1994c; Johns et al. 1998b; Kinsley et al. 1994; Nelson et al. 1998; Vernotica et al. 1996b; Vernotica et al. 1996a; Zimmerberg et al. 1992). Such effects could be mediated by cocaine's direct effects on maternal biochemical or neurobiological systems or more indirectly through numerous other behavioral systems including the stress-response, motivational, nutritional, or perceptual systems. Additionally, these systems share many common molecular, genetic, and/or epigenetic origins that could be targeted by cocaine to alter numerous systems simultaneously.

Much of the work on cocaine's direct impact on maternal behaviors has focused on its impact on the oxytocin system. Oxytocin is an important neuroendocrine system implicated in the onset of normal maternal behavior (Fuchs 1983; Pedersen et al. 1982; Pedersen et al. 1985; Pedersen et al. 1987; Pedersen et al. 1992; Pedersen et al. 1994) and therefore likely to be implicated when this behavior is disrupted. Indeed, a number of studies from our own lab have demonstrated alterations in many aspects of the oxytocin system following gestational cocaine exposure including central oxytocin levels (Elliott et al. 2001; Johns et al. 1997a), mRNA production (Jarrett et al. 2006; McMurray et al. 2008a), and receptor levels/binding (Jarrett et al. 2006; Johns et al. 2004; McMurray et al. 2008a), suggesting that this may be an important target through which cocaine may be altering maternal behavior. However, researchers have yet to find a direct mechanism through which cocaine may be altering oxytocin, and instead suggest it may be doing so indirectly through dopamine, serotonin, and/or norepinephrine (the primary reuptake inhibitor targets of cocaine in the brain).

In general, reductions in central norepinephrine levels result in disruptions in the onset of maternal behavior in rats (Rosenberg et al. 1977; Thomas et al. 1997). For example, mice lacking norepinephrine show impaired maternal behavior, which can be reversed if norepinephrine is given before parturition (Thomas et al. 1997). Norepinephrine also contributes to the release of oxytocin (Lipschitz *et al.* 2003; Russell *et al.* 2003), and norepinephrine reuptake inhibitors can increase

hypothalamic oxytocin potency (Bealer et al. 2003), though the mechanism of this is not well understood.

In addition to norepinephrine, serotonin has also been shown to modulate aspects of the oxytocin system, though the results of such research has not been as conclusive. For example, serotonin agonists may alter peripheral oxytocin release important for lactation (Bagdy et al. 1992; Bagdy et al. 1993; Bagdy 1996; Saydoff et al. 1991; Uvnas-Moberg et al. 1996) and reductions in serotonin levels may increase aggression (Coccaro 1989; Coccaro 1992; Olivier et al. 1992; Olivier et al. 1995). However, when acutely injected into ventricles, serotonin receptor agonists can reduce maternal aggression, yet have no apparent effect on maternal behavior (De Almeida et al. 1994). Thus, some debate still exists in the field as to whether serotonin positively or negatively modulates maternal behavior and aggression, though its role likely depends on the specific behavior, timing, and countless other factors.

Manipulations of the dopamine system have been more conclusively associated with alterations in various aspects of maternal behavior and oxytocinergic modulation. Dopamine agonists, particularly D2 receptor agonists, have been shown to promote the release of peripheral oxytocin (Amico et al. 1992; Amico et al. 1993; Crowley et al. 1992; Parker et al. 1992), and dopamine antagonists can disrupt pup retrieval, nest building, and motor activity in general (Byrnes et al. 2002; Giordano et al. 1990; Keer et al. 1999; Silva et al. 2001; Silva et al. 2003; Stern et al. 1999). Interestingly, dopamine reuptake inhibitors given throughout gestation (in a model similar to our own) have also been shown to enhance maternal behaviors and decrease maternal aggression (Johns *et al.* 1996; Johns *et al.* 2005b). Such results would contradict the findings we report here, were dopamine the primary mechanism of cocaine's effects on maternal behavior.

It is important to note that the involvement of the dopamine system could be due to dopamine's modulation of the oxytocin system, but could also be mediated through dopamine's involvement in motivation and reward. Dopaminergic projections from the Ventral Tegmental Area to the Nucleus Accumbens and Striatum have been the focus of countless studies investigating reward

and motivation (Ikemoto 2010), and oxytocin can modulate dopamine signalling within a number of these brain regions as well as the hypothalamus (Shahrokh *et al.* 2010). As suggested above in Figure 15, an altered motivation to care for infants following gestational cocaine exposure may also contribute to the reductions in care seen in our cocaine-treated animals. A number of studies have shown a blunting of response in reward circuits to non-drug-related rewards, enhanced responses to drug related cues, and altered responses to monoaminergic drug administration in psychomotor stimulant addicts (Kenny 2007). Indeed, there is evidence for both the anatomical ovelap of natural and drug reward systems (Kelley et al. 2002) as well as the separation of such systems (Carelli *et al.* 2000).

As indicated above, chonic drug treatment has been shown to alter the processing of other non-drug rewards and may influence the rewarding value of pups and pup-associated stimuli. In the early postpartum, dams prefer pup-associated cues over cues associated with an acute dose of cocaine (Mattson et al. 2001; Seip et al. 2007); however, it is unknown how prior exposure to chronic cocaine may alter this normal preference. It is possible that in our subjects, exposure to cocaine for the 20 days prior to birth may reduce the salience of pup cues in favor of non-pup-directed behaviors. Additionally, the rewarding salience of drugs can change over the postpartum (Seip *et al.* 2008) and it is likely that such changes also occur in the salience of natural reinforcers, such as pups. Should our cocaine-treated dams already have an altered ability to care for pups, such changes in the rewarding salience of their pups could compound any other insults to this behavioral set.

In addition to maternal behavior and aggression, oxytocin is also strongly involved in the stress response, one of the potential secondary mechanisms through which cocaine may be altering maternal behavior. Addiction is generally thought to increase stress levels in humans (Goeders 2002). Maternal stress alone can reduce the amount of care given to rodent offspring by their dams (Champagne et al. 2006) and even reduce the amount of milk produced (Lau et al. 2004). While stress levels were not measured in our dams, it seems reasonable to suggest that they may be abnormally high. Unpublished work by our lab using the forced swim and open field tests suggests this to be the

case (Williams *et al.* 2010). Specifically, our preliminary results indicate that the normal oxytocin and corticsterone response to stress seems to be dysregulated in cocaine-treated dams. In normal settings, the presence of pups can reduce the apparent stress levels of mothers (Deschamps *et al.* 2003); however, the repeated injections of cocaine or saline administered to our dams may differentially alter this normal relationship. Gestational stress alone has been shown to affect maternal care of offspring (Champagne et al. 2006). Preliminary data from our lab (Williams *et al.* 2010) indicates that the treatment paradigm used in our studies differentially alters baseline stress levels and stress responsivity in both of these groups. This is not surprising considering the saline-treated dams are also food restricted, which likely interacts with injection stress to create a differential stress response. Since cocaine and saline likely result in differential developmental effects in offspring, how these changes in maternal stress response interact with the prenatal exposure condition of the pups to determine maternal care would certainly be an excellent topic for further study considering the similarity of our cocaine- and saline-exposed pups.

As our discussion has indicated, a number of important questions remain to be addressed before we can fully understand the impact of cocaine on maternal behavior, even aside from its interaction with pup produced stimuli. With respect to the model presented in Figure 15, the data presented here and elsewhere have demonstrated that the maternal environment is altered following cocaine, and that the interaction between dam and pup is an additional contributing factor. It remains to be determined if maternal motivation, perception, etc play significant roles in altering the behavior of this half of the dyad, although cocaine's effects on maternal motivation and stress are perhaps the most interesting target for further research since findings from such studies would suggest numerous routes for behavioral intervention strategies and could also suggest numerous molecular targets for the development of pharmacotherapies.

Alterations to Postnatal Development and Behavior

Regardless of the mechanisms of cocaine's effect on maternal behavior, offspring produced stimuli and the infant's response to its environment play an important role in eliciting care. The results from Chapter 2 indicated that even when cared for by untreated mothers, cocaine-exposed pups have difficultly eliciting normal levels of care. Thus, Chapter 3 was designed to examine this aspect of the dyad and to determine if a pup's thermoregulatory ability or vocalization production was altered by cocaine exposure, which could in part contribute to more neglectful responses from their caregivers. The results from this study indicated that there were differences between cocaine-exposed pups and non-exposed pups; however, the majority of these effects were not dissociable from the effects of fetal stress/malnutrition seen in our saline control group. Since Chapter 2 indicated that cocaine-exposed pups in particular received reduce levels of care, such findings indicate that these measures were not independently responsible for the effects on maternal care as assessed here. Despite the apparent lack of a cocaine-specific effect on many of our measures, it is important to note that there are many additional pup characteristics that were not examined that may provide supplementary information to dams, such as movement patterns, olfactory, and gustatory cues (since dams carry pups in their mouths, lick them, and ingest their waste). It is likely that the few cocaine-specific effects we report here interact with these other behaviors to cause the overall phenotype of a cocaine exposed pup.

Since pups alter their own behavior in response to maternal attention, developmental disruptions in numerous physiological systems, especially the brain, could influence the pup's ability to respond normally to any mother. As discussed in Chapter 1, to date very little preclinical research has been conducted to investigate the neurodevelopmental targets of prenatal cocaine exposure. Those studies that have examined neurobiological targets have focused on the serotonin and dopamine systems, since these two systems are directly targeted by cocaine. However, the findings from such studies have been somewhat inconclusive, and depend strongly on the timing of exposure and age of assessment. Additionally, the majority of those effects reported have required very high doses of

cocaine. Although one study reported changes in the 5HT-1A receptor subtype (Johns et al. 2002) and another demonstrated an effect on serotonin release in the nucleus accumbens and striatum (Yan 2002), a number of other investigations of the serotonin system have reported no effect of prenatal cocaine on the levels of most receptor subtypes, receptor production, or degradation (Battaglia et al. 1994; Battaglia et al. 2000; Cabrera-Vera et al. 2000; Chen et al. 2004; Chen et al. 2005; Henderson et al. 1993; Vicentic et al. 2000). Similarly, investiations of prenatal cocaine's effect on dopamine system function have found minimal effects on extracellular dopamine levels (Vathy *et al.* 1993) and subtle effects on dopamine D1 receptor levels (Friedman et al. 1998), but other studies have found no change in dopamine receptor production (de Bartolomeis *et al.* 1994) and no change in dopamine release within mesolimbic brain regions (de Bartolomeis *et al.* 1994; Phillips *et al.* 2003). Such an ambiguous picture reinforces the possibility that prenatal cocaine's long term effects on behavior are the result of extremely subtle changes in brain function, although future studies may determine otherwise.

Like all behaviors, the behaviors studied in this dissertation are controlled by a number of brain regions, although the specific regions used depend slightly upon the age of the subject. This is perhaps due to changes in motivating mechanisms behind each behavior. For example, as suggested in Chapter 3, ultrasonic vocalization production at very young ages is likely the product of laryngeal braking; however, as the pup grows older, social factors become their predominant determinant, thus shifting the neurobiological control of this behavior from the periaqueductal grey and brainstem to more socially-integrated regions such as the hippocampus and hypothalamus. Of course, despite this shift, motor control of the larynx and respiratory system still relies upon the brainstem. Similar shifts likely exist in the control of thermal behavior. The shifting of neurobiological control of each behavior presents a potentially interesting target for the study of developmental insults, and may help explain why behavioral differences that are seen early in life may not be apparent later in life, and vice versa. Such effects emphasize the importance of longitudinal study designs. Had we looked at these measures at later time points we may have seen cocaine-specific differences.

In an attempt to expand upon the current body of literature in a highly translational way, we have begun an investigation of prenatal cocaine's impact on brain development in rats using advanced neuroimaging techniques. While the results from this study may not be able to suggest molecular targets of prenatal cocaine exposure, it does allow for an anatomical and organizational examination of the entire brain. Such data will be of tremendous benefit to the field, as it will highlight additional brain regions related to behaviors of interest through association and suggest anatomical regions and connections of circuits for future studies to examine. The preliminary results from this study suggest that there are anatomical and organizational alterations to the hippocampus, olfactory bulb, and colliculi (McMurray *et al.* 2010); however, only a portion of this project has been completed, so interpretation of these results should be done with caution.

Aside from central nervous system effects, prenatal cocaine exposure likely affects numerous other physiological systems such as the metabolic, renal, and cardiovascular systems. Many of these systems have been discussed elsewhere in this dissertation (see Chapters 1, 3 and 4); however, it is worth noting that prenatal cocaine may also have effects on the developing peripheral nervous system, which could contribute to all of the previously mentioned physiological systems. The peripheral nervous system delivers information to the brain from sensory systems and peripheral organs, and in turn delivers information to these systems from the brain. Historically, psychologists have considered the central and peripheral nervous systems as important players in the determination of behavior; however, recent studies have tended to focus on the central nervous system and we have perhaps lost sight of the importance of peripheral action. A return of focus on the peripheral system may provide a number of revelations regarding prenatal cocaine's effects on pup behavior very early in life, such as crying and thermoregulation.

When considering the effects of prenatal cocaine exposure we report here in light of the Polyvagal Theory (Porges 2009), it seems reasonable to suggest that alterations in infant arousal may be due to alterations in polyvagal balance. The Polyvagal Theory was introduced to explain the different functions of the two primary source nuclei of the vagus: the nucleus ambiguus and the dorsal

motor nucleus. The fibers originating in the nucleus ambiguus are thought to be responsible for respiratory sinus arrhythmia, while those fibers originating in the dorsal motor nucleus are responsible for enacting survival strategies, such as fighting, fleeing, or freezing. Despite the functional dichotomization, both of these pathways terminate on the same region (sinoatrial node), which is thought to control cardiac function. Based on this theory, shifts in respiratory sinus arrhythmia and heart rate could be explained by the independent actions these two pathways. Therefore, polyvagal theory would suggest that the branch of the vagus originating in the nucleus ambiguus would inhibit the otherwise acceleratory sympathetic nervous system input to the heart when attention and social engagement are adaptive, and withdraw this inhibition when fighting or fleeing are adaptive. Since thermogenesis is highly dependent on the metabolism of brown fat, shifts in vagal output could have dramatic implications on this process. Similarly, infant crying is in part determined by the level of arousal achieved, again suggesting that vagal imbalance could result in an over- or under-aroused infant. Interestingly, not only is the start and stop of a crying bout potentially determined by vagal efferents (Nakazawa *et al.* 1997), but also the pitch, amplitude, and numerous sonographic characteristics (Porter *et al.* 1988). Interestingly, good vagal tone has been suggested by numerous investigators to be an excellent predictor of infant resilience following prenatal cocaine exposure (Mehta et al. 2002; Sheinkopf et al. 2006a; Sheinkopf et al. 2007). Such findings would suggest that in addition to its central and peripheral nervous system effects, the intersection between the peripheral and central nervous systems to be a likely target of prenatal cocaine exposure.

The results presented here suggest a strong similarity between the effects of fetal cocaine and those of fetal stress. Fetal stress can be vasoconstrictive resulting in hypoxia, increase levels of stress-associated molecules (corticosterone, adrenalin, etc.), and may be capable of activating the developing infant's HPA axis (Charil et al. 2010; Lester et al. 2009). Prenatal stress has also been associated with numerous metabolic effects (for review see (Tamashiro et al. 2010)), and fetal hypoxia has been shown to alter cardiac development, potentially affecting thermoregulatory capacity (Patterson et al. 2010). As discussed in the Chapter 1, these effects can have long reaching

implications on development. Specific to this dissertation, research on the impact of prenatal stress on vocalizing behavior has been somewhat inconclusive, with some studies showing an increasing number of ultrasonic vocalizations (Harmon et al. 2009; Williams et al. 1998), others showing a decrease (Morgan et al. 1999) on PND 14, and effects on earlier time points are unknown. The period and intensity of stress seems to be a determining factor and it is difficult to determine the intensity of the stressor used in our models. Thus, interpretation of the data presented here in light of this is more challenging.

In addition to fetal stress, postnatal factors (neglect, sickness, etc.) can also result in stress to the developing pup, which can, in turn, affect behavior. For example, relevant to the current dissertation, repeated isolation from the mother as might occur in our cocaine group has been shown to alter vocalizing behavior (Goodwin et al. 1994). Our own anecdotal evidence might suggest that repeated isolation from the mother is stressful, at least in humans; however, no physiological measures of stress were collected in this study. Such effects should have been minimized to an extent in the current dissertation by not testing the same animal repeatedly. It is relevant to note though, that even though no experimental isolation was conducted, cocaine-treated mothers have been shown to isolate their pups more than other groups without our involvement. The interaction between such postnatal stressors and any existing prenatal factors may have contributed to the similarity between the saline and cocaine groups.

Mortola and colleagues (Mortola et al. 1998) have reported that early postnatal stress, and to some extent prenatal stress, can alter thermogenic capacity. Their results indicated that prenatal stress paradoxically decreased interscapular BAT tissue levels, while at the same time increasing thermogenic capacity. The authors attributed this finding to changes in body weight (which would alter the surface to volume ratio) and potential changes in blood oxygen carrying capacity, resulting in an increase in thermogenic efficiency. Interestingly, intermittent maternal separation as seen in our cocaine-treated dams would provide intermittent cold stimuli, which can stimulate BAT growth and thermogenic capacity (Cannon et al. 1983; Skála et al. 1974). Additionally, the thermogenesis can

also be affected directly by stress (Benedek et al. 1983; Tornatzky et al. 1993), which is likely due to likely activation of the preoptic thermoregulatory neurons (Vellucci et al. 1995) and likely increased sympathetic activation of BAT through increasing norepinephrine turnover (Yoshida et al. 1994). In light of the increased interscapular and back temperatures on PND 3 reported in this dissertation, it would be most interesting to determine if BAT levels are decreased in our animals. Such results would suggest an even stronger relationship between the effects found in the cocaine and saline exposed groups.

The results presented here regarding alterations in early life behavior following prenatal cocaine exposure describe only two behaviors used by pups that communicate information to dams: temperature and vocalizations. These two behaviors appear to be only marginally affected by cocaine at the time points examined in this study, and those effects were similar to the effects of prenatal stress alone. Thus, the picture presented by our data fall in line with current views of prenatal cocaine, which suggest that its effects are relatively subtle in comparison to fetal alcohol and other developmental disorders, and likely involves interactions between both prenatal and postnatal factors. Clearly, this was not an exhaustive investigation of cocaine's effects on early life behavior, but the two behaviors examined are at the core of numerous physiological processes, such as cardiac and respiratory regulation. Numerous other behavioral and anatomical differences remain to be addressed (see Figure 15). However, the lack of highly significant differences in the important behaviors examined here would suggest that if these underlying processes are affected by prenatal cocaine, the effects are extremely subtle and restricted to very specific behavioral sets.

Conclusions

While this dissertation has answered very specific questions about the impact of cocaine on maternal-infant interactions, the results from these studies leave questions unanswered. Though Chapter 2 may have demonstrated that cocaine affects the ability of dams to care for pups and that cocaine-exposed pups have difficulty in eliciting care from their dams, Chapter 3 failed to find highly significant differences in several behaviors of these pups thought to be important for elicitation of care. Thus, further exploration of other stimuli or combinations of other stimuli that may impact maternal response is required to answer these questions. The sensory capacity of the rat differs dramatically from those of the human, therefore the characteristics of infants that we as a species consider most important may not be as relevant to rodents. Chapter 4 was designed to address numerous aspects of this system; however, was unable to effectively do so. Despite this, Chapter 4 was able to show that cocaine is indeed present in urine from young pups. While this finding has numerous implications regarding the developmental impact of cocaine, it is unknown how this may interact with the olfactory and gustatory systems of rat dams to alter the quality of care these pups are able to elicit. It is highly unfortunate that the one chemical in urine known to play a role in maternal stimulation, dodecyl propionate, was not able to be measured in our samples.

One strength of the studies conducted in this dissertation is their translational relevance. For example, it is possible to measure many of the same endpoints in humans that this dissertation has addressed in rodents. The interactions between human cocaine-using mothers and their infants have been previously studied by many (Strathearn et al. 2010b), reporting similar reductions in the quality of care we report here. An interesting extension of this study is ongoing in human mother infant dyads to determine the salient characteristics of infants that drug using and non-drug using mothers typically attend. Such studies will prove immensely valuable to our understanding of both how cocaine may be altering both parties of the dyad and perhaps lead to future intervention studies. While our results suggest that these measures may not be as sensitive a tool as is needed to investigate many subtle developmental disorders in rodents, they may have more clinical value. A particularly valuable

finding from this dissertation is that maternal stress, which may come from many sources in the human environment, are likely to have a large impact on the developing child and should be highlighted as an important area of future research.

In conclusion, the data we present here add to the literature characterizing the impact of cocaine on mother infant interactions. These data suggest that cocaine-induced decreases in maternal care are likely the result of its effects on both parties involved in the dyad (mother and offspring). While we were unable to detect considerable differences in pup behavior following prenatal cocaine exposure, our results demonstrate that rat dams are able to detect differences between cocaine-treated and untreated pups and alter their behavior towards them. Continued research on the relevant physiological and behavioral effects of cocaine on rat pups may supplement our understanding of the factors that determine high quality interactions between mothers and pups. Considering the predominantly social nature of both humans and rodents, understanding the factors that contribute to successful dyad interactions can have tremendous implications in other fields of psychology and public health.

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