PRENATAL STRESS DOES NOT SIGNIFICANTLY ALTER SINGLE-TRIAL CONDITIONED PLACE PREFERENCE IN ADOLESCENT RATS

by

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A Thesis
Submitted to the
Graduate Faculty
of
George Mason University
in Partial Fulfillment of
The Requirements for the Degree
of
Master of Arts
Psychology

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Date: December 9, 2011

Fall Semester 2011
George Mason University
Fairfax, VA

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DEDICATION

This is dedicated to my parents, who love and support me unconditionally.

ACKNOWLEDGEMENTS

I would like to thank Dr. Robert Smith, Dr. Craig McDonald, and Dr. Marjorie Battaglia for their dedication and guidance throughout the thesis process. I would also like to thank my lab mates: Katie Taylor and Kelsey Brown for their invaluable help in the preparation of this project.

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ABSTRACT

PRENATAL STRESS ALTERS SINGLE-TRIAL CONDITIONED PLACE PREFERENCE IN ADOLESCENT RATS

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Early environmental and behavioral experiences can affect development during adolescence and even adulthood. Prenatal effects can result in lasting changes on the nervous system and behavior. Gestational stress has been shown to lead to an increased vulnerability to substance abuse and addiction disorders. Exposure to stress is associated with sensitive periods of vulnerability that also uniquely contributes to drug abuse vulnerability. Compulsive drug use can increase due to a highly reactive HPA-axis, which can be dysregulated by prenatal stress exposure (Andersen & Teicher, 2009). While the results of studies done with other drugs of abuse suggest that animals exposed to prenatal stress are more vulnerable to substance abuse, the effects of prenatal stress on nicotine exposure is unknown, particularly on initial nicotine experiences during adolescence. Indeed, research on the effects of prenatal stress on adolescent behavior and development is scarce. As adolescence is the most common age for initial nicotine use, and as adolescence is a sensitive period where reinforcing effects of nicotine are stronger

than in adults, we carried out an experiment to determine the effects of prenatal stress on adolescent nicotine reward using a rat model. The conditioned place preference (CPP) paradigm, widely used to assess the rewarding effects of abused drugs in rodents [Bardo & Bevins, 2000], is ideal for modeling initial responsivity to nicotine. In this experiment, pregnant female rats underwent either restraint stress [45 min 3X daily, P14-P21], or no stress. Adolescent male offspring underwent a four-day, single-trial nicotine CPP procedure in a conditioning apparatus consisting of 2 distinct sides. The results show that prenatal stress altered the posttest time of nicotine place preference in adolescence. This suggests the prenatal stress may actually be acting on anxiety and that could be altering the conditioned place preference results.

1. INTRODUCTION

What happens to a fetus in utero can affect development and can also have an effect later in life. These effects can result in permanent alterations on nervous system structure and function. Early environmental stress is one of the most influential things that can occur during gestation which, can result in later psychopathology and alterations of the central nervous system. In humans, children of mothers who have been exposed to gestational stress have alterations in motor behavior, brain morphology, and an increased risk of developing behavioral disorders such as attention-deficit hyperactivity disorder, sleep disorders, cognitive dysfunction, increased anxiety, schizophrenia, depression, and substance abuse disorders (Kippin, Szumlinski, Kapasova, Rezner, & See, 2008; Campbell, Szumlinski, & Kippin, 2009).

Animal studies investigating the effects of prenatal stress have yielded similar results. Animals whose mothers have been exposed to stress during late gestation can develop increased anxiety, learning and memory impairments, altered circadian rhythm functioning, and impaired sexual functioning (Kippin et al., 2008). Neuroendocrine and behavioral hyperresponsiveness to stress, along with an enhanced responsiveness to the behavioral activating and reinforcing properties of drugs of abuse have also been found (Campbell et al., 2009).

Prenatal stress can also have significant consequences for brain development, which ultimately leads to the behavioral impairments discussed previously. Prenatal stress has been shown to decrease the number of granule neurons, induces an absence of hippocampal neurogenesis, reduce the density of nitric oxide producing neurons in the dentate and hippocampus, and causes expanded lateral amygdaloid nucleus, the area in which learned fear is encoded (Yang et al., 2006). An enhanced synaptic efficacy of the hippocampal CA1 region and the effects of acute stress on synaptic plasticity reactivity in offspring has been seen in offspring whose mothers experienced significant gestational stress (Yang et al., 2006). Restraint stress specifically, has been shown to result in the denser packing of synaptic vesicles and increased mitochondrial area in mossy fiber terminals (Gordon, 2002).

Alterations in the neuroendocrine system and several neurotransmitter systems can also occur in prenatal stress offspring. Stress hormones that reach the fetus from maternal circulation include catecholamines, CRH, and adrenal steroids (Weinstock, 2008). CRH induces the release of glucocorticoids from the adrenal gland of the fetus by activating CRH type 1 receptors (Weinstock, 2008). Many animal studies have shown that prenatal exposure to stress elicits a marked increase in plasma glucocorticoid levels in the mother (Yang et al., 2006). However, in the fetus, prenatal stress has been shown to result in a decrease in glucocorticoid receptors in the hippocampus (Mabandla et al., 2008). The glucocorticoids are most abundant in the hypothalamic CRH neurons and the pituitary where they serve to regulate the negative feedback of CRH release (Weinstock, 2008). Other stress hormones can also be altered by exposure to stress during gestation

including cortisol. In rats exposed to repeated stress during days 14-21 of gestation, elevation of free-circulating cortisol was found in both the fetus and the mother (Weinstock, 2008). The elevation of circulating cortisol seen in the mother can eventually lead to alterations in the development of the fetal hypothalamic-pituitary-adrenal axis, which serves as the stress circuit in the brain (Weinstock, 2008). The HPA axis in the rat develops between days 13 and 15 and can react to maternal COR on day 15 of gestation (Weinstock, 2008). The regulation of the feedback response to stress of the HPA axis is mediated through the glucocorticoids (Weinstock, 2008). Abnormal feedback regulation of the HPA axis and an increased sensitivity of the HPA axis has been found in rats exposed to prenatal stress (Weinstock, 2008). Overall, prenatal stress can result in significant dysregulation of the HPA stress circuit.

Neurotransmitter systems are also affected by exposure to stress during gestation. Increased noradrenaline levels have been seen immediately after exposure to a footshock stress (Yang et al., 2006). Also, a greater release of dopamine in the prefrontal cortex in response to prenatal stress has been found in rodents (Weinstock, 2008). In addition, an increase in acetylcholine release in the hippocampus after mild stress has been seen (Yang et al., 2006). Alterations of dopamine and glutamate receptors in various brain regions can consequently lead to a change in the development and formation of the corticostriatal and corticolimbic pathways (Weinstock, 2008). Once again, stress exposure during gestation can lead to permanent changes in the function of neurotransmitter systems.

Gestational stress, as well as stress in general, has been shown to lead to an increased vulnerability to substance abuse and addiction disorders. Exposure to stress is associated with sensitive periods of vulnerability that also uniquely contributes to drug abuse vulnerability (Andersen & Teicher, 2009). Compulsive drug use can increase due to a highly reactive HPA-axis, which is consequently dysregulated by prenatal stress exposure (Andersen & Teicher, 2009). Early life stress could potentially be more selective for the hippocampus, which enhances contextual responding to drug-related cues. It can also increase dopamine activity in the nucleus accumbens, resulting in a state of anhedonia that can predispose individuals to drug-seeking behavior (Andersen & Teicher, 2009). Moreover, early environmental stress is also selective for the prefrontal cortex and increases vulnerability to drug-associated cues (Andersen & Teicher, 2009). Glucocorticoids that are influenced by prenatal stress exposure modulate behavioral and neurochemical signaling effects of drugs (Thomas, Hu, Lee, Bhatnagar, & Becker, 2009). These points provide evidence for the effects of prenatal stress having an influence for subsequent vulnerability to drugs of abuse.

Prenatal Stress and Drugs of Abuse

Several animal model studies have shown that prenatal stress influences response to various drugs of abuse. These drugs of abuse include cocaine, amphetamines, morphine, alcohol, and nicotine.

Repeated maternal restraint stress is the most popular stress paradigm used to investigate the effects of prenatal stress on reactivity to drugs of abuse. The Kippin laboratory (2008) assessed locomotor activity induced by a novel environment along

with, the acquisition and maintenance of self-administration and reinstatement to cocaine. Female and male rats were mated overnight. At gestational day 14, pregnant rats were exposed to repeated restraint stress. In adult offspring, locomotor activity in response to novelty was assessed following exposure to cocaine. Cocaine-induced self-administration, extinction, and reinstatement of cocaine seeking were also assessed using an FR-1 schedule of reinforcement. Microdialysis and HPLC were used following behavioral testing to examine neurochemical responses to noncontingent cocaine exposure. PNS rats displayed an enhanced locomotor response to novel environments relative to controls. Furthermore, PNS rats also showed alterations in nucleus accumbens dopamine, serotonin, and glutamate functioning compared to controls (Kippin et al., 2008). Prenatal stress increased cocaine seeking in rats that had a history of self-administration during extinction training and during reinstatement. Therefore, rodents exposed to prenatal stress are more likely to engage in drug-seeking behaviors and relapse (Kippin et al., 2008).

A study conducted by Thomas and colleagues (2009) also examined the effects of prenatal stress and cocaine. They specifically examined sex-specific susceptibility to cocaine. Once again, male and female rats were housed together until the detection of a copulatory plug by vaginal lavage. On day 15 of gestation, pregnant rats were exposed to a repeated restraint stress similar to the one used by the Kippin laboratory. Self-administration training began at PND 55-60. Both female and male offspring were trained to self-administer cocaine on a fixed-ratio 1 schedule of reinforcement. In order to track the progression of the estrous cycle in female rats, vaginal lavage was performed at

the conclusion of each session. Animals who were not trained in self-administration were tested for sensitization to cocaine by examining locomotor activity after acute and repeated injections of cocaine (Thomas et al., 2009). The results of this study indicated that PNS males self-administer more cocaine than non-stressed controls. Also, the amount of cocaine self-administered increased across test sessions as the doses increased. On the other hand, PNS females but not males, exhibited a hyperresponsive reaction to cocaine's psychomotor-activating effects (Thomas et al., 2009). Behaviorally, the results of this study show that there are sex differences in the response of PNS to cocaine and these modulate the sensitivity of rats to both the reinforcing and psychomotor-sensitizing effects of cocaine (Thomas et al., 2009).

Based on the results of these studies prenatal stress clearly alters reactivity and susceptibility to cocaine addiction. One constant present in both of these studies was the prenatal stress paradigm, repeated restraint stress. While the stress paradigm remained consistent some of the results within the two studies varied. For example, Thomas et al. (2009) found that males self-administered more cocaine than non-stressed rats however, Kippin et al. (2008) did not find this effect. This may be due to the difference in dosages used for self-administration training. Kippin et al. (2008) used a lower dose of cocaine than the Thomas laboratory. It is possible that the sensitivity to cocaine is more likely to be induced by higher doses of cocaine. However, the hyperresponsive reaction to cocaine's psychomotor-activating effects was seen in both studies. Meaning, that PNS reliably elicits increased locomotor activity at many different cocaine doses. Therefore,

being exposed to stress during gestation increases susceptibility to cocaine use and addiction (Kippin et al., 2008; Thomas et al., 2009).

The effects of prenatal stress and vulnerability to alcohol addiction have also been examined. Darnaudery et al. (2007) assessed early environmental stress on alcohol preference in female rats. Male and female rats were paired together and vaginal smears were examined. Pregnant female rats were exposed to a repeated restraint stress paradigm. At 2 months old, alcohol preference was assessed in a two bottle choice paradigm. Alcohol preference was assessed for two weeks and then four weeks during the aversive procedure. In order to account for individual variability in the spontaneous preference for alcohol, the impact of an aversive procedure alcohol preference was also assessed (Darnaudery et al., 2007). The aversive procedure consisted of a single exposure to an inescapable footshock, followed by 3 weekly exposures as a situational reminder. The prenatal stress rats did not differ from controls in terms of free-access alcohol intake. However, PNS females that initially displayed a high preference for alcohol further increased their intake when subjected to intense stress (footshock) (Darnaudery et al., 2007). These results indicate that prenatal stress may increase vulnerability to alcoholism in adulthood.

The effects of prenatal stress on alcohol preference and sensitivity to alcohol exposure were assessed by Van Waes and colleagues (2010). Sprague-Dawley rats were bred in their laboratory. Pregnant females were exposed to repeated restraint stress from day 11 of gestation through delivery. The offspring was exposed to a chronic alcohol procedure beginning on PND 28. Alcohol preference was assessed using a two- bottle

choice procedure in adolescence. In adulthood, rats were exposed to a chronic forced alcohol treatment where the only drink present was alcohol. Following the forced alcohol a two-bottle choice procedure was used during which, the rats had the choice between tap water and various alcohol concentrations (2.5%, 5%, or 10%). Alcohol deprivation was also assessed; by temporarily raising the voluntary intake of alcohol when alcohol is was reinstated after a period deprivation (Van Waes et al., 2010). Alcohol preference was also evaluated in PNS rats when alcohol was in competition with a 1% sucrose solution. Locomotor reactivity to novelty and anxiety-related behavior was assessed in the elevated plus maze. Prenatal stress did not affect spontaneous alcohol preference or the motivation for alcohol after chronic alcohol exposure. However, chronic alcohol intake was found to suppress the hyper-reactivity to novelty associated with PNS (Van Waes et al., 2010). Also, chronic alcohol exposure did not have an effect on parameters of stress or anxiety. Overall, the results indicated that PNS rats did exhibit a higher sensitivity to the effects induced by chronic alcohol exposure and PNS exacerbated locomotor reactivity to novelty (Van Waes et al., 2010).

To date, these are the only studies reporting on the effects of prenatal stress on alcohol the vulnerability to alcohol addiction and alcohol preference. Together these studies provide evidence for an increased susceptibility to alcohol abuse for those previously exposed to early environmental stress. Once again, these studies are consistent with their use of repeated maternal restraint stress as the prenatal stress paradigm. But, differences in initial preference for alcohol was seen. Darnaudery et al. (2007) found an initial high preference for alcohol in a specific subset of PNS rats, while no initial

preference for alcohol was found in the Van Waes laboratory. The two studies used different concentrations of alcohol. Therefore, Darnaudery et al. (2007) using a lower dose may have elicited the preference for alcohol since at lower doses alcohol is more appealing (Van Waes et al., 2010). Both studies also did not find a difference between PNS rats and controls in spontaneous alcohol consumption. However, drug intake does not necessarily affect motivation for the drug (Van Waes et al., 2010). In fact, just inhalation of the alcohol vapors can induce drug dependence (Van Waes et al., 2010). Therefore, PNS may lead to a higher sensitivity of the brain reward systems to alcohol.

Yang et al. (2006) conducted a study examining the effects of prenatal stress exposure on morphine addiction. It has been shown that stress facilitates the initial acquisition and maintenance of drug self-administration and can elicit relapse. Yang and colleagues investigated whether prenatal stress can enhance addictive behavior in offspring, specifically, the impact of PNS on conditioned place preference to morphine. Male and female Wistar rats were housed together until the detection of a sperm plug which was defined as gestational day 1. The pregnant rats were exposed to chronic stress, each day, from days 13 to 19 during pregnancy. Stress consisted of footshocks for 30 minutes a day. After weaning, rats were exposed to morphine conditioned place preference in adulthood. They were also tested in the forced swim test to assess their depressive-like behavior. This study found that prenatal stress enhanced the addictive behavior in adult offspring, prenatal stress increased morphine- induced conditioned place preference. Also, the prenatal stress rats were found to exhibit more depressive-like

behaviors than controls (Yang et al., 2006). This finding is not surprising since an increase in depression is seen in children and rodents exposed to stress in utero.

This study provided important information on the effects of prenatal stress and addictive behavior. However, there are several limitations to this study. First, the stress paradigm to which the mothers were exposed was very different from most of the studies examining prenatal stress exposure and drug abuse. The majority of the research conducted in prenatal stress uses restraint stress which has an advantage over footshock stress because, it can affect the fetus indirectly through direct contact with the mother (Ward & Weisz, 1984; Carboni et al., 2010). Also, the amount of morphine administered to the rats during conditioning was 10 mg/kg. This is a relatively large dose of morphine for rodents. While conditioned place preference was reliably induced using the dosage, this dose may not be equal to that used in humans. In contrast to the study conducted by Yang and colleagues, many other prenatal stress studies used varying doses of the drugs of abuse (Kippin et al., 2008; Campbell et al., 2009). Exposing PNS rats to different doses of the drug is important to determine how much increased sensitivity to drugs PNS rats exhibit.

While the effects of prenatal stress have been examined at length for other drugs of abuse such as cocaine, alcohol, and morphine, research in nicotine is virtually non-existent. There are only two studies, which have assessed prenatal stress and the later effects on nicotine exposure.

Koehl, Bjijou, Le Moal, and Cador (2000) conducted a study examining the preexposure of rats to prenatal stress on nicotine-induced locomotor activity. The

hypothesis of the study was that prenatal stress would increase the sensitivity of the adult offspring to the locomotor effects of nicotine. Adult virgin Sprague-Dawley females were house individually for an entire estrous cycle in the presence of a sexually experienced male. Pregnant rats were then randomly assigned to prenatal stress and control groups. Prenatal stress was conducted and the pups were weaned at 21 days of age and then tested a 3 months. Horizontal locomotor activity was tested in response to novelty and to different doses of nicotine (0.0, 0.1, 0.2, and 0.4 mg/kg). The rats were first exposed to novelty for 2 hours. Two days later, they received different does of nicotine following a 1-hour period of habituation to the activity cages. The results of this study found an enhanced sensitivity to nicotine following exposure to prenatal stress. Specifically, they found a dose-dependent increase in locomotor activity at doses of nicotine up to 0.4 mg/kg. Meaning, that prenatal stress modifies the pathway involved in the psychomotor-stimulant effects of nicotine (Koehl et al., 2000). The manipulation of the early environment can have effects on the later behavior of the pups including increased sensitivity to drugs of abuse.

In contrast to the behavioral research conducted by Koehl and colleagues, the other study that examined the effects of prenatal stress and nicotine was purely an anatomical study. Carboni, Barros, Ibba, Silvagni, Mura, & Antonelli (2010) used microdialysis to examine the effects of prenatal restraint stress on catecholamine relsease in the rat prefrontal cortex. This study evaluated whether DA and NA extra-cellular concentration in the prefrontal cortex of prenatal stress rats and controls were affected by amphetamine and nicotine. Pregnant rats that had been assigned to either a prenatal stress

or control group were exposed to restraint stress three times a day for forty-five minutes each. The stress paradigm took place between the 14th and 21st days of pregnancy. Microdialysis was conducted in both adolescent and adult rats. They measured the basal output of both NA and DA prior to administration of the drug. Once the basal output had reached stable levels, the rats were exposed to a single challenge dose of the test drug. After, histological analysis was performed. They found that nicotine stimulated DA output in both adolescent and adult PNS rats were not significantly different from controls. However, they did find that DA output was significantly higher in adolescent rats compared to adults (Carboni et al., 2010). These results suggest that PNS does not influence nicotine response in the PFC associated with DA transmission. However, the higher response seen in adolescents overall may be due to the established phenomenon of adolescents being more sensitive to the effects of nicotine (Carboni et al., 2010). Prenatal stress did not alter the response of NA to nicotine in adolescence however; it was significantly lowered in adults. Summarily, the results of this study suggest the alteration of DA and NA output in the PFC response to nicotine.

Previously, the Carboni laboratory conducted a study with the same methodology except they were examining dopamine and noradrenaline release in the nucleus accumbens shell. The nicotine effect in the prenatal stress rats was higher than in controls in both adults and adolescents (Silvagni et al., 2008). PNS produces neurobiological changes in DA transmission in response to nicotine administration. Nicotine reinforcement is linked to the activation of nicotinic acetylcholine receptors (nAChR) in the mesocorticolimbic dopamine pathway that projects from the ventral tegmental area to

the nucleus accumbens and the prefrontal cortex (Koob & Le Moal, 2006). Nicotine conducts its actions by binding to the nAChR complex and the cholinergic input to the mesolimbic dopamine pathway may provide a system through which nicotine can increase dopamine release (Koob & Le Moal, 2006). Therefore, an increase in DA output could be interpreted as a higher nicotine reinforcing effect and therefore, a higher vulnerability to nicotine in PNS animals (Silvagni et al., 2008). They also found that nicotine-stimulated NA output was higher in adult PNS rats than in controls. In adolescent rats, the concentration of NA was lower than in controls. Once again, suggesting alterations in the transmission of NA in the nucleus accumbens. Another mechanism in which nicotine can activate the mesolimbic dopamine system involves an excitatory role for NMDA receptors (Koob & Le Moal, 2006). Since nicotine clearly alters the output of NA in the nucleus accumbens shell it is probable that nicotine is acting indirectly on NMDA receptors to activate NA neurons, which project to the hypothalamic paraventricular nucleus and the amygdala (Silvagni et al., 2008). These projections then may be involved in either the nicotine stress response or in drug craving (Silvagni et al., 2008). Overall, stress has been shown to produce a sensitization of the reward system that leaves those who have experienced significant amounts of stress more responsive to drugs of abuse and more likely to develop an addiction (Silvagni et al., 2008).

Although the research conducted by Carboni and colleagues suggests that prenatal stress alters the response to nicotine in both adolescents and adults there is no behavioral data to confirm these findings. Neuroanatomical data is extremely important to

understand the way drugs of abuse alter the functioning of neurotransmitters and various brain areas. Nevertheless, behavioral experiments are also needed to further examine the behavioral consequences of the alterations in brain functioning. Therefore, future research needs to focus on behavioral experiments assessing the effects of prenatal stress and nicotine exposure.

Conditioned Place Preference

Conditioned place preference (CPP) is a paradigm used to measure drug reward in laboratory animals. It demonstrates the ability of an environment to become associated with 'drug wanting behavior', acquiring conditioned incentive properties. In theory, CPP reflects the animal's preference for a particular situation due to the association between that context and a drug stimulus (Tzschentke, 2007). In an unbiased CPP design, subjects are randomly assigned to drug conditioning exposure on either side of a two or three-chambered apparatus. A biased CPP procedure takes into account that rats display an initial side preference. The bias is incorporated into the procedure, and establishes a baseline preference measure in which drug pairings only occur in the non- preferred chamber. This paradigm demonstrates the behavioral effects of addictive drugs on the VTA- mediated reward system.

CPP has been used to demonstrate drug preference differences across age groups. Belluzi found that while adult rats did not exhibit a preference for nicotine-paired environments, rats that were conditioned during adolescence exhibited a significant preference for the nicotine-paired compartment after a single conditioning trial (Belluzi, 2004). Brielmaier found that CPP was established following a single pairing of nicotine

in early adolescent but not adult animals. Age differences were also seen in the response to a nicotine challenge injection, in which adult-pretreated rats demonstrated tolerance to the locomotor depressant effects of a low dose and adolescent-pretreated rats to a higher dosage of nicotine (Brielmaier, 2007).

Brielmaier, McDonald, and Smith (2011) also examined the effects of stress on adolescent conditioned place preference. As stated previously, adolescence is a time of increased sensitivity to the rewarding effects of nicotine. It is possible that the increase of nicotine use exhibited by teenagers is also affected by stress by potentially augmenting the rewarding properties of nicotine (Brielmaier et al., 2011). They examined this relationship by exposing adolescent rats to a single episode of footshock 24 hours before CPP. Conditioned place preference was tested with three different doses of nicotine (0.2, 0.4, and 0.6 mg/kg). Brielmaier and colleagues found that animals that were exposed to stress acquired nicotine CPP at all three nicotine doses. These results suggest that a stressor can enhance the rewarding properties of nicotine seen in adolescence (Brielmaier et al., 2011).

To date, there is no research examining the effects of prenatal stress on the rewarding properties of nicotine exposure in adolescence. Specifically, there are no behavioral experiments examining this phenomenon. While the results of studies done with other drugs of abuse suggest that animals exposed to prenatal stress are more vulnerable to substance abuse, the effects of prenatal stress on nicotine exposure is unknown. There is also not much previous research examining the effects of prenatal stress on adolescent substance abuse. Since adolescence is a proposed critical period

where vulnerability to addiction is increased and the rewarding properties of the drugs are enhanced, research in this area is necessary.

Furthermore, stress exerts its maximal effects on the prefrontal cortex in adolescence. The increasing development of the prefrontal cortex may result in increased vulnerability to stress during adolescence (Andersen & Teicher, 2009). Also, high levels of glucocorticoid receptors are expressed in the PFC during adolescence, which may render the PFC even more susceptible to stress effects (Andersen & Teicher, 2009). Adolescence is a critical window of vulnerability for addiction to drugs. The full effects of early environmental stress may lie dormant until adolescence. Early environmental stress could potentially program an altered trajectory of development that continues through adolescence and into young adulthood (Andersen & Teicher, 2009). Therefore, research delving into the effects of prenatal stress on susceptibility to drugs of abuse in adolescence is important.

Elevated Plus Maze

Novel stimulation often evokes fear in the rat, it has also been shown that rats naturally exhibit more avoidance behaviors in open, elevated alleys than in closed alleys (Hogg, 1996). This is the primary basis for the development of the elevated plus maze (EPM). The EPM has been validated in its use as an animal model of anxiety. Forced or voluntary passage into the open arms of the elevated plus maze has been associated with elevated corticosterone, increased freezing behavior, and an increased production of feces. All of these behaviors are indicative of increased anxiety (Hogg, 1996). In order to examine the anxiety producing behaviors in the elevated plus maze the entries made into

the open arms and the time spent in the open arms are recorded (Padovan and Guimaraes, 2000). Results consistently indicate that spending more time in the open arms of the elevated plus maze suggests more fear and anxiety in the rat.

Current Experiment

In order to examine the effects of prenatal stress on the rewarding properties of nicotine in adolescence, rodents exposed to prenatal restraint stress were tested on nicotine conditioned place preference (CPP). CPP is a widely used behavioral paradigm to measure drug reward in laboratory animals (Vastola et al., 2002). CPP has been successfully established in adolescent animals, which permits the testing of animals in a drug-free state, which does not require extended periods of training (Vastola et al., 2002). In addition, the relation of anxiety in the mothers who are exposed to stress and their offspring's development of conditioned place preference was examined. I hypothesize that the rats exposed to prenatal stress will be more vulnerable to nicotine abuse and will find nicotine more rewarding than those not exposed to prenatal stress. I also hypothesize that the mother's that will experience stress will exhibit more anxious behavior in the elevated plus maze and that the scores computed in the elevated plus maze will closely correlate will the conditioned place preference difference scores.

2. METHODS

Subjects

Nulliparous female Sprague-Dawley rats around 65 days of age (n=24) were group housed with 4 female rats per cage. Twelve sexually inexperienced male rats were also group housed in groups of 4. The rats were given two weeks to acclimate to their new housing environment. Following habituation to their new environments, the females were paired with one male rat and left housed until the detection of a sperm plug. The day of detection was designated as gestational day 0 (GD 0). Pregnant rats were housed separately in clear 12"x18" cages Plexiglas cages lined with Tek-Fresh bedding with *ad libitum* access to water and standard laboratory rat chow. Vivarium conditions were maintained at 21-23° C, relative humidity ~55-60% under a 12:12 light/dark cycle.

Experimental Group Assignments

There were a total of four experimental groups, each with about 12 animals per group. An a priori power analysis concluded that a total of 48 animals were needed to find a moderate effect size. Animals were housed according to their group identfication which were as follows: Prenatal Stress-Nicotine; Prenatal Stress- Saline; No Stress-Nicotine; No Stress- Saline. The first drug assigment pertains to the stress condition, and the second is the drug assignment which relates to the CPP conditioning drug.

Materials

Nicotine hydrogen tartrate was purchased from Sigma Chemical Company (St. Louis, MO). All drugs will be administered at an injection volume of 1 mL/kg body

weight. Saline and nicotine were administered subcutaneously between the shoulder blades (0.5 mg/kg). The dose level was chosen based on previous experiments in our lab, which have indicated that this dose reliably produces place preference in adolescents (Brielmaier et al., 2007). To minimize discomfort of the younger animals, 26 ½ gauge needles will be used for all injections. Nicotine was dissolved in 0.9% NaCl and dose levels are expressed as free base.

Prenatal Stress took place in a Plexiglas, transparent cylinder (internal diameter 6 cm) that has adjustable lengths (15-18 cm).

CPP conditioning was carried out in a 2-chambered conditioned place preference insert (Med Associates, VT) located in a very dimly lit (4-6 1x) testing room. The apparatus consists of 2 plexiglas chambers each measuring 21x42x30 cm. One chamber consists of black walls with a stainless steel mesh floor and black tray paper lining, whereas the other consists of white walls with a stainless steel rod floor and white tray paper lining. A black removable guillotine door separates the two chambers. A camera was mounted above the apparatus and records each trial, and data were acquired using Videotrack software (Viewpoint, Montreal, QC, Canada).

The EPM apparatus (Kinder Scientific, Poway, CA) is arranged in a plus-shaped configuration with two arms (50.2 cm x 10.8 cm) surrounded by black Plexiglas walls and two opposing open arms (50.2 cm x 10.8 cm). The apparatus is elevated 86 cm off the ground. Non-maze related cues are limited in the room to minimize impact on behavior and light levels are set at approximately 3 lux in the open arms. Testing lasts for 5 minutes and anxiety-like behavior is measured by time spent in the open arms.

Procedure

Pregnant female dams were exposed to repeated restraint stress or left undisturbed in their home cage. The PNS group was transferred to an experimental room where the selected stress paradigm was performed. The restrainers were fitted closely to body size for three periods of 45 minutes each per day (9:00, 12:00, 4:00) between the 14th and 21st (included) day of pregnancy. This type of stress originally described by Ward & Weiz (1984) was chosen because it influences the fetus indirectly through direct stress on the mother.

At P1 (postnatal day 1) pups were sexed, weighed, and when possible the litter was culled to equal numbers of males and females until there are 8 total pups per litter. Daily weight of the offspring was recorded for the duration of the study. Control and PNS pups remained with their biological mother until they were weaned at P21. Only male offspring were used for conditioned place preference. A maximum of 5 male pups were placed in each cage and left undisturbed until they began CPP training. Only 2 pups from each litter were used in CPP training to avoid litter effects.

At postnatal day 28 male adolescent pups (n=40) exposed to stress in utero were tested in a biased place conditioning procedure. Meaning that animals were tested for natural chamber preference and conditioned with the drug in their non-preferred chamber. Testing consisted of three phases: a pretest day, conditioning sessions, and a posttest day. On the pretest day, animals were placed in individual wire hanging cages and allowed to habituate to the testing room for a period of approximately 20 minutes. Each animal was then placed into the testing apparatus for 15 minutes with the guillotine door

removed to allow free access between the chambers. Unconditioned preference was determined by recording the time spent in the white chamber of the apparatus, which was defined as the part of the trial during which all four paws were located in the white chamber. Placement was counterbalanced within groups such that half of the rats started in one chamber and half starts in the other. Between trials on all experimental days, both sides of each insert were cleaned with 70% EtOH and tray paper changed to remove odor cues. During the first conditioning session, prior to testing, animals were weighed before placement in the wire cages and again habituated to the CPP testing room for 20 min. Following habituation to the testing room, experimental animals were injected with 0.5% mg/kg nicotine (s.c.) and immediately placed in their initially non-preferred side or injected with saline (s.c.) and immediately placed in their initially non-preferred chamber. During the second conditioning day, all animals were injected with saline and immediately placed in their initially preferred side. On both conditioning days, animals were placed in their respective chambers facing away from the removable door. Conditioning sessions lasted 15 min, and the order of sessions (nicotine or saline first) were counterbalanced within the groups. On the posttest day, a 15 minute drug-free posttest were conducted to determine chamber preference following drug exposure. As with the pretest day, the guillotine door was removed to allow free access between the chambers, placement in either the black or white chamber was counterbalanced within groups and preference was determined for each animal by recording the time spent in the white chamber of the apparatus.

Approximately 2-4 weeks post-weaning, female rats that were exposed to restraint stress during pregnancy were tested in the elevated plus maze in order to assess the effects of restraint on anxiety-like behavior.

Data Analysis

Conditioned place preference results were analyzed using a 2 (drug treatment: nicotine or saline) x 2 (stress: prenatal stress or no stress) analysis of variance (ANOVA). The elevated plus maze results were analyzed using Pearson correlations. All statistical analyses were analyzed using SPSS 17.0 statistical processing software.

3. RESULTS

Statistical Analysis

Nicotine CPP Acquisition

To determine acquisition of CPP, a difference score was computed for each animal by subtracting time in seconds spent in the white chamber on the pretest from the time spent in the white chamber on the posttest. The scores were analyzed using a 2 x 2 ANOVA (pretreatment stress: prenatal stress, no stress; conditioning drug: nicotine, saline) for significant interactions between CPP scores and pretreatment stress exposure.

Elevated Plus Maze

The relationship of prenatal stress on the mother's anxiety level as measured by EPM score and its effects on the acquisition of conditioned place preference of the offspring was examined. The scores were analyzed using a Pearson correlation coefficient (elevated plus maze score; conditioned place preference difference score).

Outliers

Outliers were coded, and excluded from analysis, if the difference scores were above or below three standard deviations from the mean. Using these criteria, 2 animals were dropped from analysis, and a total of 47 animals were used in the current experiment, for a total of 45 data points. Table 1 illustrates the total number of subjects per experimental group.

Table 1. Subject Totals Per Experimental Group

Group	*Total N*
Prenatal Stress/ Nicotine CPP Conditioned	14
Prenatal Stress/Saline CPP Conditioned	12
No Stress/ Nicotine CPP Conditioned	11
No Stress/Saline CPP Conditioned	8

Conditioned Place Preference

There was a significant main effect of conditioning drug on CPP induction, F (1, 41) = 17.203, $p < .001$. Mean comparisons illustrate that animals conditioned with nicotine had significantly higher difference scores, and thus spent more time in the non-preferred, drug paired chamber (M= 59.64) than animals conditioned with saline (M= -43.95). These results signify that the nicotine-conditioned animals did form preference to the nicotine-paired chamber. Main effects are illustrated in figure 1.

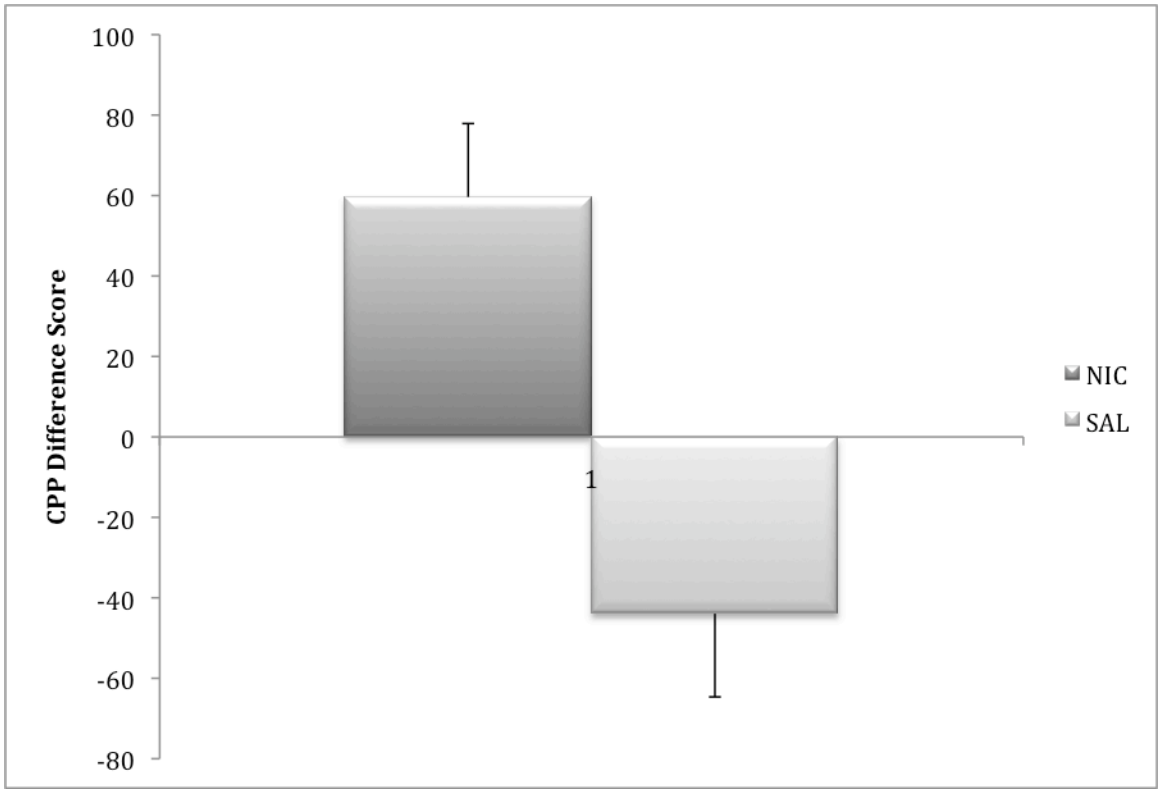

Figure 1. Conditioned place preference difference scores. Nicotine conditioned animals demonstrated higher difference scores compared to animals conditioned with saline, $p <$ 0. 01.

There was a significant main effect of stress on CPP induction, $F(1,41) = 5.920$; $p < .05$. Mean comparisons illustrate that animals exposed to the stress condition, regardless of drug conditioning had significantly higher difference scores, and thus spent more time in the non-preferred, drug paired chamber (M=37.46) than animals who were not exposed to the stress condition (M=-19.05). These results suggest that the animals exposed to the prenatal stress condition were more likely to form a preference to the nicotine-paired chamber. Main effects are illustrated in figure 2.

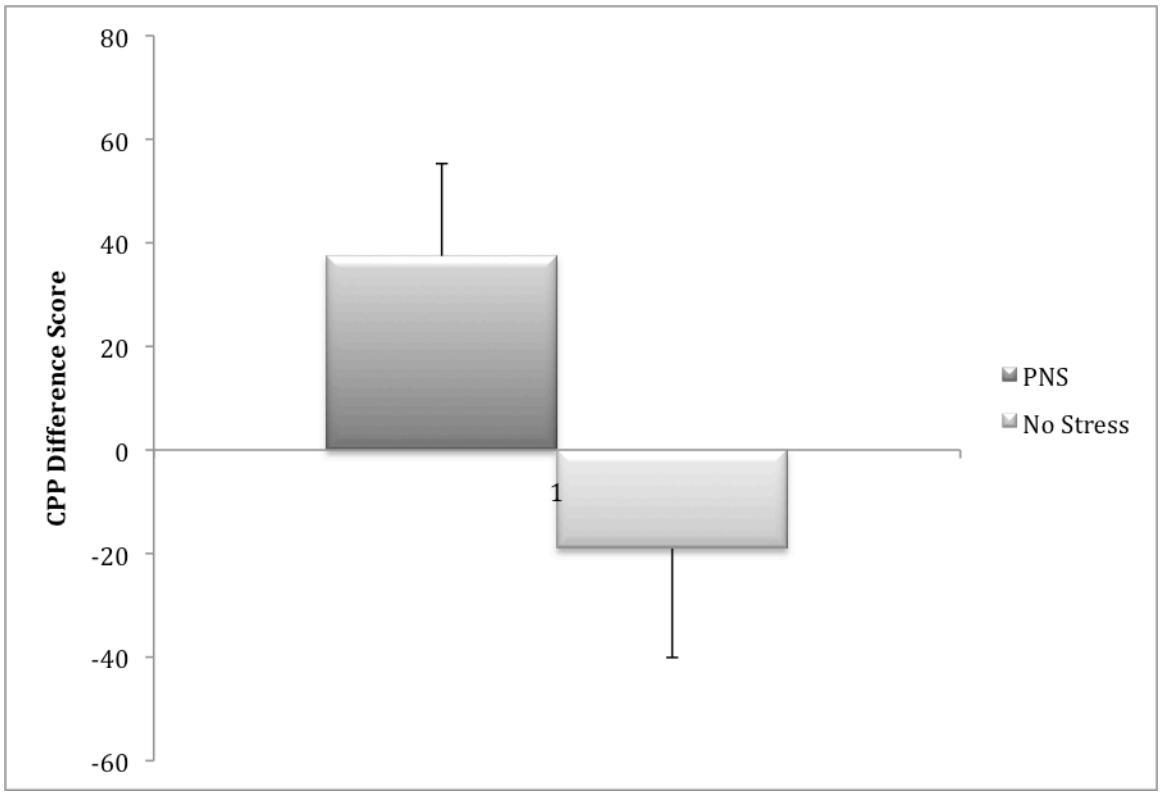

Figure 2. Conditioned place preference difference scores. Animals who were exposed to prenatal stress prior to conditioned place preference demonstrated higher difference scores compared to animals that were not exposed to stress, $p < 0.05$.

There was a significant interaction effect between the stress condition and drug pretreatment condition $F(1,41)= 3.402$, $p < .05$. This indicates that CPP conditioning with nicotine during adolescence is affected by stress exposure in utero. Contrasts revealed that animals spent in the no stress saline conditioned spent significantly more time in the black chamber than animals in the prenatal stress nicotine conditioned group, $F(3,41)=7.62$, $p < .05$. Animals in the prenatal stress saline group spent more time in

white chamber than animals in no stress saline group, $F(3,41)= 7.62$, $p < .05$. There was also a difference present in the amount of time animals in the no stress nicotine group spent in the drug-paired chamber compared to the animals in the no stress saline condition, $F(3,41)=7.62$, $p < .05$. Additionally, there was a significant difference in the amount of time animals exposed to prenatal stress conditioned with nicotine spent in the drug-paired chamber compared to Interaction effects are illustrated in figure 3.

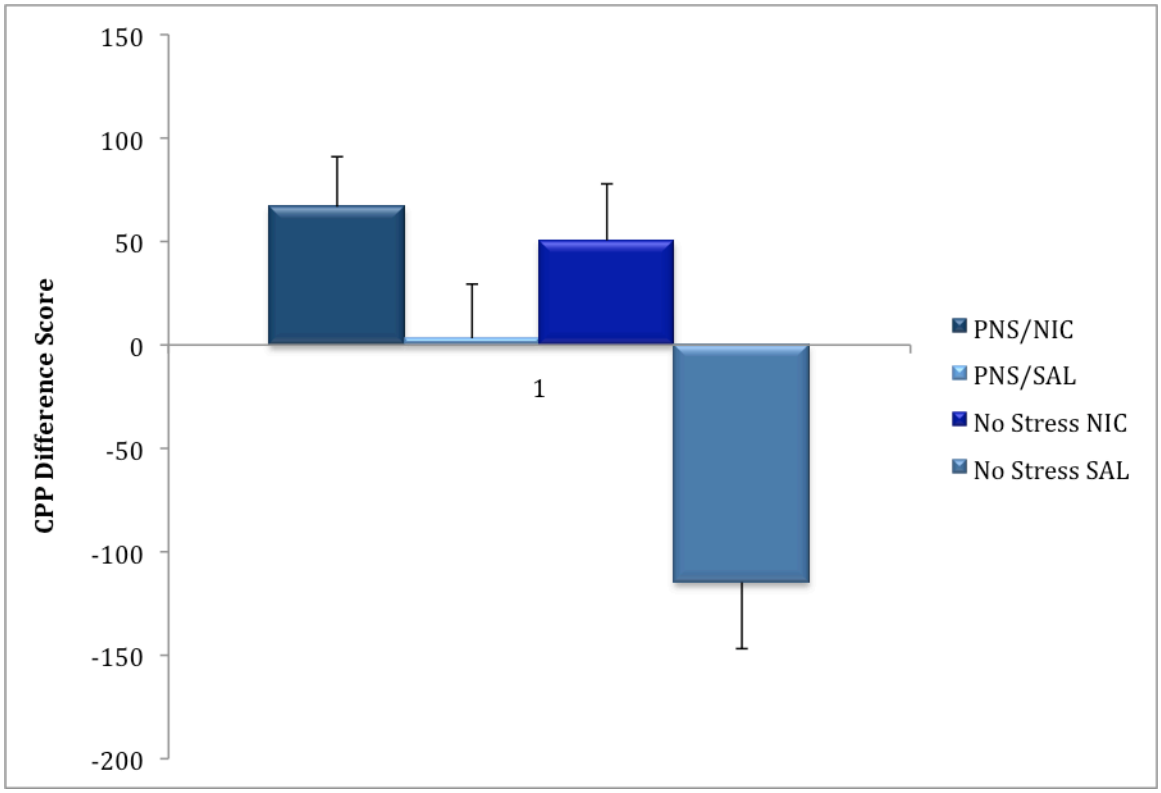

Figure 3. Difference scores on conditioned place preference. Animals who were conditioned with nicotine and exposed to prenatal stress exhibited higher difference scores compared to animals in the comparison groups, $p < 0.05$.

Elevated Plus Maze

There was no significant correlation between the mother's time spent in the open arms of the elevated plus maze and her offspring's conditioned place preference difference score, (r^2= -.042, p > .05). More time spent in the open arms of the elevated plus maze suggest that the animal is less anxious than an animal that spends the majority of its time in the closed arms of the elevated plus maze. There was also no significant correlation between the mother's time spring in the closed arms of the elevated plus maze and her offspring's conditioned place preference difference score, (r^2= -.069, p > .05). The results indicate that there was no correlation found between the mother's level of anxiety due to prenatal restraint stress and her offspring's likelihood to form conditioned place preference. The correlations are illustrated in figure 4.

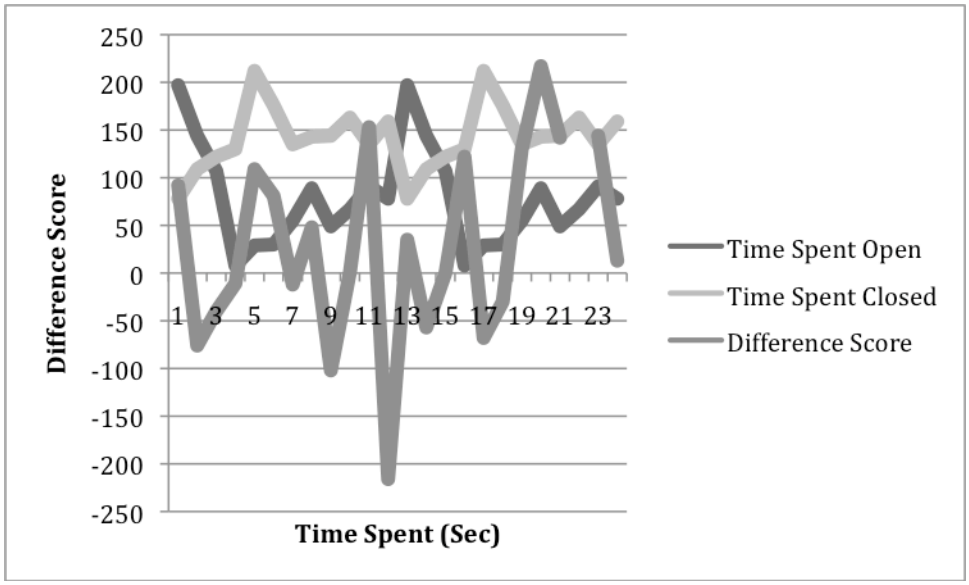

Figure 4. Time spent in the open and closed arms of the elevated plus maze correlated to her offspring's difference score. The mother's time spent in both the closed and open arms of the elevated plus maze are not correlated to their offspring's conditioned place preference, $p > 0.05$

4. CONCLUSION

The current study found that male adolescent rats that were exposed to prenatal stress did not exhibit a stronger place preference to nicotine using a single trial-conditioning paradigm. Animals in both nicotine-conditioning groups formed a place preference suggesting that conditioned place preference did occur in all animals. Based upon the current results, it can be inferred that prenatal stress may be having an effect on anxiety and contribute to the tendency for the differences seen in the conditioned place preference. This study also found that the mother's anxiety level measured 2-4 weeks after weaning does not correlate with their offspring's likelihood to form nicotine place preference.

CPP Induction

Results of the current study indicate that animals that were conditioned with nicotine spent more time in the drug-paired side of the chamber. This result is regardless of the prenatal stress condition. This confirms the results of previous studies that adolescent animals can form conditioned place preference with a single nicotine pairing (Brielmaier et al., 2007). Once again, suggesting that animals find nicotine more rewarding in the adolescent stage.

This study failed to confirm the hypothesis of the current study that prenatal stress alters the rewarding properties of nicotine in adolescence. However, prenatal stress does alter the posttest time of nicotine conditioned place preference. It is possible that prenatal stress alters a mechanism similar to anxiety, as the animals exposed to prenatal stress

conditioned with saline did not exhibit the negative difference scores seen in the animals not exposed to stress conditioned with saline. This alteration in anxiety also potentially contributed to the small difference seen in animals exposed to prenatal stress conditioned with nicotine and the animals not exposed to stress also conditioned with nicotine. Therefore, that prenatal stress could be acting on nicotine's ability to counteract stress-induced anxiety or by activating nicotine's conditioned anxiolytic effects (Brielmaier et al., 2010). This confirms the results of several studies that examined the effects of prenatal stress on anxiety. A study conducted by Estanislau & Morato (2006) found that animals exposed to prenatal stress and tested in the elevated plus maze at P45 spent more time in the open arms of the maze and entered the open arm ends of the maze more often than the animals tested at P30. Indicating that prenatal stress augments the effects of anxiety in the elevated plus maze, causing there to be an inverse relationship of between prenatal stress and anxiety during adolescence. The results of this study and the current experiment propose that prenatal stress can affect nicotine conditioned place preference by activating the anxiolytic properties of nicotine and the inverse relationship between prenatal stress and anxiety.

There is also a possibility that there is an effect of prenatal stress occurring in the current study, which is suggested by the results of the current study. However, the effect of prenatal stress could be masked by the current measure of reward. Since the conditioned place preference paradigm is a biased model of reward the effect may not present itself in the current study. The results support the idea of an inverse relationship between prenatal stress and anxiety. Therefore, using another measure of anxiety or

reward could potentially reveal a prenatal stress effect that cannot be detected with the current paradigm.

Elevated Plus Maze

Although a relationship between the stressed mother's anxiety level and their offspring's likelihood to form nicotine place preference is not supported by the current experiment, there remains the possibility that alternate testing paradigms may find a significant effect. One of the reasons this part of the experiment was conducted was to ensure that the prenatal restraint stress procedure was inducing anxiety as a measurable behavior. However, since this experiment failed to conduct the elevated plus maze experiment on both stressed and non-stressed mothers, anxiety of the mothers could not be compared directly. Therefore, the stressed mother's anxiety level had to be compared to their offspring's CPP induction. A direct comparison to both maternal groups would be ideal, however, due to time and equipment restraints this was not possible.

There are no previous studies to date, which examined the effects of prenatal stress on the mother's level of anxiety. However, a study conducted by Estanislau and Morato (2005) assessed the effects of prenatal stress in the elevated plus maze. They used both footshock and restraint stress prior to examining anxiety-like behavior in the elevated plus-maze. The experimenters found that the percentage of entries into the open arms of the maze were significantly reduced by restraint stress and that rats exposed to prenatal restraint stress exhibited even lower values than the other control and experimental groups. These results suggest that prenatal stress can lead to responses that have been modified by the stress exposure in utero (Estanislau & Morato, 2005). Prenatal

stress increases the inhibitory avoidance naturally seen in animals who are placed in a novel exploratory environment, which can be interpreted as, prenatal stress leading to an increase in generalized anxiety (Estanislau & Morato, 2005). Similar brain changes seen in the amygdala of prenatally stressed rats and in fMRI images of patients who suffer from generalized anxiety disorder further confirms these findings (Estanislau & Morato, 2005). Furthermore, suggesting that if tested, the mothers who were exposed to prenatal stress would exhibit higher levels of anxiety in the elevated plus-maze compared to non-stressed mothers.

Another limitation seen in the elevated plus maze portion of the experiment is the amount of time after birth the testing occurred. Due to a busy testing schedule, the necessity of sharing testing rooms, and the inability to plan when births occurred the testing of the stressed mothers occurred at several different times after weaning of her pups. While all mothers were tested in the elevated plus maze within a 2-4 week period after weaning, a more consistent testing schedule could potentially yield different results. It is possible that allowing more time to pass after weaning and after the stress occurred could have affected the correlations seen with the offspring.

Future Directions

This study determined that there is a relationship between prenatal stress and nicotine conditioned place preference in adolescence. There are several interesting extensions of this experiment that should be explored in the future.

Prenatal stress can lead to a variety of different behavioral and neurobiological consequences. There is evidence that prenatal stress effects are mediated by maternal

hormones that are passed through the placenta to the offspring (Estanislau & Morato, 2006). However, the activation of many of these effects on the offspring depends upon the age when the subjects are tested (Estanislau & Morato, 2006). It is possible that many neurobiological and behavioral effects of prenatal stress can only begin to be detected during a sensitive period of development such as, adolescence or adulthood. Perhaps prenatal stress affects the rat pups more strongly during a different period of development and could lead to more defined results. Therefore, the inclusion of a late adolescent and adult comparison group would be good addition to the study. Estanislau and Morato (2006) conducted a study examining the effects of prenatal stress on exploratory and anxious behaviors in the elevated plus-maze using various different age groups. They tested groups in the elevated plus-maze at 30, 45, and 60 days old. Estanislau and Morato (2006) found that prenatal stress effects in the elevated plus-maze emerge at late adolescence only, as mentioned previously the late adolescent males explored the open arm end of the maze more than the control groups. Social behaviors in unfamiliar environments change throughout adolescence (Estanislau & Morato, 2006). Therefore, throughout adolescence behaviors are changing due to hormone fluctuation. By exploring the open arm end of the maze the late adolescent males are showing an increase in risk-taking behavior, which is not seen in the early adolescent or adult groups. In human adolescents, elevated risk-taking behaviors are associated with drug abuse and such problems are more prevalent in males and in late adolescence (Estanislau & Morato, 2006). Therefore, with the addition of a late adolescent group, the effects of prenatal stress on the rewarding properties of nicotine may be revealed. It is possible that the age

group tested in the current experiment was too young to exhibit the effects of prenatal stress on drug abuse. The results of this study suggest that examining the effects of prenatal stress on nicotine conditioned place preference using both a late adolescent and adult comparison group could potentially yield a different outcome. Potentially, even demonstrating a facilitative role of stress in the initial rewarding effects of nicotine during late adolescence, a period during which many smokers begin their use of cigarettes (Brielmaier et al., 2010). Moreover, this study also suggests that prenatal stress can lead to an increase in behaviors associated with drug abuse although in a different age group than currently explored. For this reason, use of various different age groups in future proposals is worth exploring.

Another interesting extension of this project due to the proposed interaction with anxiety could be to examine anxiety directly. Since it is proposed that prenatal stress could be influencing anxiety-like behaviors in the adolescent animals, examining their behavior in an animal model of anxiety would be worthwhile. In order to understand the mechanisms that are at work in the current experiment testing using the elevated plus maze or light-dark box could provide further evidence.

The scope of the current project failed to include an investigation of neural connectivity following prenatal stress and CPP induction, or a study of neurobiological changes following poly-drug exposure. Use of neurobiological assays such as Golgi stains, CORT analysis, and c-fos analysis could detect changes induced by prenatal stress and nicotine conditioned place preference in the adolescent brain. While there have been studies examining the neurobiological correlates and prenatal stress and nicotine

exposure, there are no current studies which examines the relationship between prenatal stress and nicotine conditioned place preference in the brain.

Another extension of this experiment would be to examine the effects of prenatal stress and the reinforcing effects of poly-drug use. Recently, there has been an increase in poly-drug use in both adolescent and adult populations. However, very little research examining the effects of poly-drug use on the brain and behavior has been conducted. Since adolescence is often a period of experimentation with drugs as well as, a time of synaptic pruning, growth, and change in the brain it is especially important to understand how drug use affects adolescence. Extending the current study to incorporate other drugs of abuse will be extremely beneficial for the research community.

Furthermore, integrating the results of the current project along with previous similar studies with other drugs of abuse into a public service campaign would also potentially be helpful. To date, expectant mothers are taught to try and minimize their stress levels by learning coping mechanisms, exercise such as prenatal yoga and Pilates, and psychotherapy if necessary. All of these coping mechanisms is important for normal, healthy prenatal development. However, expectant mothers are not provided with the information that significant levels of stress could have an effect on their child's well being in adolescence or even adulthood. Developing a plan to disseminate this information to the public could be extremely beneficial for expectant mothers and their future children. Perhaps motivating them to be even more aware of the stress they are under and researching ways they can attempt to be stress-free.

The results of this study demonstrate that prenatal stress exposure can have lasting effects on the behavior of the offspring well into adolescence. Prenatal stress potentially has an effect on the anxiety level in the adolescent animals, which potentially altered the posttest scores of nicotine conditioned place preference. The altered anxiety could be contributing to the enhanced CPP seen in the current study. Adolescence is known as a period of increased vulnerability to substance abuse due to the dysregulation of the mesolimbic dopamine pathway that occurs. Prenatal stress leads to enhanced HPA axis stress responsiveness (Estanislau & Morato, 2005). The enhanced HPA stress response along with the increased rewarding properties of drugs of abuse during adolescence leads to a likelihood that prenatal stress could further increase the rewarding properties of drugs of abuse. However, the results of this study did not find that rats exposed to stress in utero form a stronger preference for nicotine than rats that were not stressed. Once again, this could be due to the early age that was tested in the current study. Overall, the results of the current study suggest that prenatal stress has an effect on nicotine conditioned place preference, modulating a mechanism similar to anxiety.

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CURRICULUM VITAE

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