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Sex differences in behavioral and intracellular responses to cocaine

By

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Abstract

Sex differences in behavioral and intracellular responses to cocaine

By

Arbi Nazarian

Advisor: Professor Vanya Quinones-Jenab

Female rats are more sensitive to cocaine-induced behaviors than male rats. However, the underlying mechanisms involved in this sensitivity are not well understood. The purpose of this proposal is to assess the role of dopamine receptors (D1 and D2) and their related signal transduction pathway in the sex differences in cocaine-induced reward, locomotion, and sensitization. To study the role of D1 and D2 receptors in cocaine reward, a conditioned place preference (CPP) paradigm was utilized. Specifically, the D1 antagonist SCH 23390, and the D2 antagonist eticlopride were administered to male and female rats during cocaine conditioning to block the development of CPP. All doses of SCH 23390 blocked cocaine CPP in male rats, whereas in female rats, only the low and the moderate dose of the drug blocked cocaine CPP. Eticlopride, on the other hand, did not to block cocaine CPP in male or female rats.

To measure the role of signal transduction mechanisms in sex differences to cocaine, protein kinase A (PKA) and cAMP response element binding protein (CREB) levels were measured after an acute injection of cocaine. Female rats given acute cocaine demonstrated a longer lasting and more robust locomotor enhancement than male rats. Interestingly, female rats had higher basal levels of PKA in the nucleus accumbens (NAc) than male rats. After acute cocaine, accumbal CREB phosphorylation was enhanced in both sexes, but the enhancement was much shorter lasting in female rats than in male rats

(5min vs. 30min). Cocaine did not alter CREB phosphorylation in the caudate-putamen (CPu) of either sex, nor were PKA levels changed in CPu or the NAc.

Sex differences after chronic cocaine administration was studied along with measurements of PKA and phosphorylated CREB. Overall, male rats developed sensitization, while female rats exhibited behavioral tolerance to cocaine-induced locomotor behaviors. No differences in PKA or phospho-CREB levels were detected in the NAc or CPu between chronic cocaine, acute cocaine, or saline treated male and female rats.

Based on the present findings, dopamine receptors have a limited role in the sex differences to cocaine's response, whereas intracellular signaling components appear to be contributing to the sex difference observed in the behavioral measurements.

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Chapter 1: Introduction

I. Background:

A. History of cocaine abuse:

Cocaine is an active alkaloid found in the leaves of *Erythroxylon coca*, a tree indigenous to Peru and Bolivia. The drug has been used as a psychostimulant among the people of Colombia for 2,000-5,000 years [135]. In the United States however, its use remained relatively limited until the late 1800s. Around this time, cocaine began to see heightened popularity when a young physician by the name of Sigmund Freud advocated its use as a treatment for a multitude of personality disorders and even morphine addiction [113]. It wasn't long before reports of cocaine addiction threatened its new founded popularity, and by 1894, the American Medical Association was beginning to question its use [135]. Finally in 1914, the use of cocaine was banned by the Harrison Narcotic act.

B. Overview and Rationale:

Since the ban of this substance as a psychostimulant and potentially addictive substance, its use has fluctuated affecting all classes and races of people. Today, there is a lack of understanding about the unequal distribution of cocaine use between the sexes. In 2002 the National Household Survey on Drug Abuse reported that 33% of the 1.3 million Americans that currently use cocaine were women [166]. Although males are more likely than females to have an initial opportunity to use drugs, there seems to be no difference in the progression to intense drug use following the initial use [172;173]. Hence, sex differences in the pattern of drug abuse may be circumstantial thereby providing males with greater opportunities to progress from initial to habitual use.

In the past decade, there has been a growing interest in the study of the differential properties and actions of cocaine between males and females. Early studies suggested that females react differently to cocaine than males. For example, female rats tend to be more sensitive to cocaine-induced locomotion than male rats [66;174]. Since then, many investigators have utilized behavioral and neurochemical techniques to study sex differences in cocaine responding in human and non-human animals.

The objective of this thesis is to further understand the sex differences in cocaine-induced behaviors by using both behavioral and neurobiological paradigms. Firstly, the present study will measure the involvement of dopamine receptors in the sex differences in cocaine-induced behaviors. Experiments using D1 and D2 receptor antagonists will be conducted to study the role of these receptors in cocaine-induced locomotor activity and cocaine reward using the place preference paradigm. Secondly, acute and chronic cocaine-induced sex differences will be further studied by measuring acute locomotor activity, sensitization of the locomotor response, and stereotypic behaviors. Lastly, sex differences in cocaine reaction will be assessed at the intracellular signal transduction level, where a number of signal transduction proteins (i.e., protein kinase A and CREB) will be measured. The ultimate goal of this thesis is to determine whether sex differences in cocaine-induced behaviors are mediated via the intracellular signal transduction mechanisms.

C. Dopamine Synthesis:

Catecholamines are subdivided into different transmitters. By far the most important catecholamines are dopamine, norepinephrine, and epinephrine. Due to the limitation of this project, dopamine will be the only neurotransmitter under study.

Dopamine is synthesized from the precursor aromatic amino acid tyrosine [123]. There are two steps involved in the synthesis of dopamine from tyrosine. The first step in dopamine synthesis is the hydroxylation of L-tyrosine to L-DOPA by the catalyzing enzyme tyrosine hydroxylase. Tyrosine hydroxylase is the rate-limiting enzyme in catecholamine synthesis. It is believed that the maximum velocity of tyrosine hydroxylase is slower than that of any of the other enzymes in the synthetic pathway [119]. As a result, the activation of tyrosine hydroxylase governs the overall rate of formation of all of the catecholamines. The second step in dopamine synthesis involves the conversion of L-DOPA into dopamine by the enzyme L-amino acid decarboxylase, which can increase its activity very rapidly.

Upon synthesis, dopamine is packaged into vesicles and transported to the terminals for storage [184]. Vesicular dopamine is released by the influx of Ca^{2+} due to membrane depolarization. Once released into the synaptic cleft, dopamine can be taken up into the terminals via the dopamine transporters [60;70]. The dopamine transporter recaptures dopamine shortly after its release, thus modulating its concentration in the synapse and affecting its interaction with dopamine receptors [89;154].

D. Effects of cocaine on the dopaminergic system:

Cocaine binds directly to monoamine transporters and prevents their reuptake, increasing the concentration of these neurochemicals in the synapse [68]. Cocaine's effects on dopamine uptake occur within the mesocorticolimbic pathway, which has been implicated as having a primary role in mediating the reinforcing properties of many drugs of abuse including cocaine, amphetamine, and opioids (Figure 1) [85]. The limbic circuitry that mediates the rewarding effects of acute psychostimulant administration

include dopaminergic cell bodies that project from the ventral tegmental area (VTA) to the frontal cortex, nucleus accumbens, and caudate putamen [86;146;147]. The amygdala has also been implicated in drug reward and cocaine-seeking behaviors [6;29;131]. Cocaine's effects on dopamine neurons in the pre-frontal cortex, ventral pallidum, and olfactory tubercles also appear necessary for cocaine reinforcement [58;72;85;91;150].

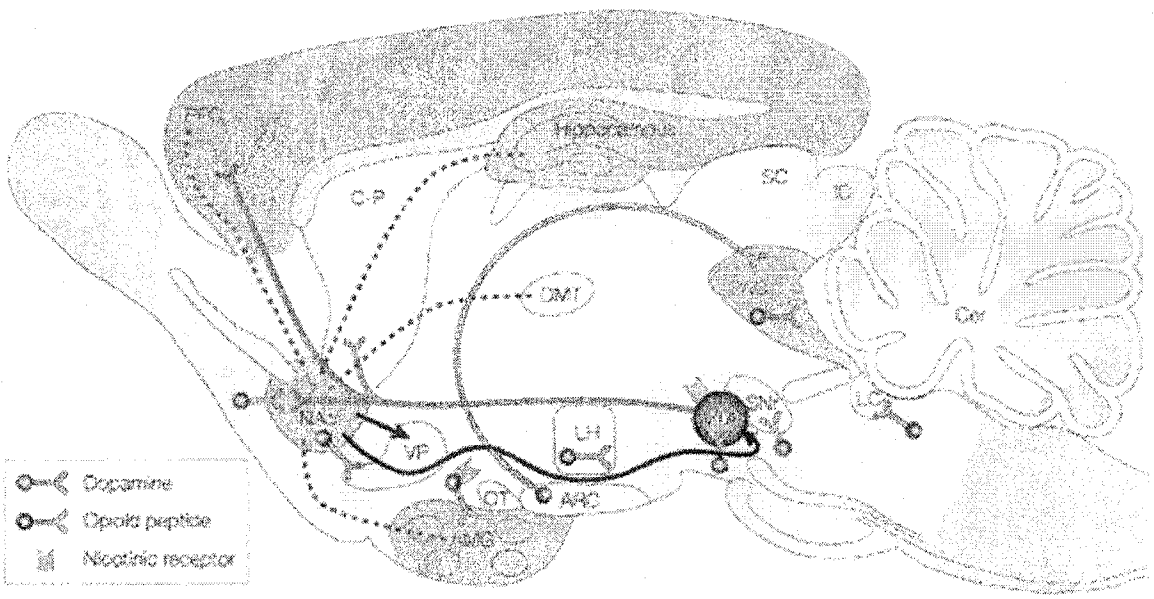

Figure 1: The reward circuit. Dopaminergic projections from the VTA terminating in the nucleus accumbens, caudate putamen, and the frontal cortex are of primary interest in drug abuse research. (Adopted from Nestler, 2001).

Cocaine potentiates dopaminergic neurotransmission within the mesocorticolimbic pathway. The VTA projections to the nucleus accumbens have been postulated to play a pivotal role in the rewarding effects of cocaine. It has been shown that cocaine increases extracellular dopamine concentrations in the VTA and the nucleus accumbens of freely moving rats, but with time course differences in dopamine release[142]. Under normal physiological conditions in the VTA, there are GABAergic medium spiny neurons that express D1 dopamine receptors. These GABA neurons project to the VTA and form inhibitory synaptic connections, where dopamine acting on these GABA afferents inhibits VTA neurons [23]. It has been shown that chronic cocaine alters the normal physiological state by attenuating the GABA mediated inhibition, which in turn potentiates neuronal activity [16]. This potentiation of neuronal firing acts to increase dopamine release in the nucleus accumbens. It is generally assumed that changes within this neural circuit after chronic use of drugs of abuse underlies the behavioral changes we observe. There is a plethora of data indicating semi-permanent cellular adaptations in these limbic nuclei after repeated administration of drugs of abuse [126]. It is still not well delineated as to how alterations in the VTA and nucleus accumbens contribute to the craving and relapse seen in chronic drug users.

E. Dopamine receptors and their role on cocaine-induced behaviors:

There are two classes of dopamine receptors, D1-like (D1 &D5) and D2-like (D2, D3, & D4), based on their pharmacological response to various compounds. D1 receptors are found throughout the forebrain and are concentrated in the substantia nigra, nucleus accumbens, caudate putamen, entopeduncular nucleus, olfactory tubercle, the island of Calleja, amygdala, and hippocampus[36]. D2 receptors are found in the nucleus

accumbens, caudate putamen, olfactory tubercles, island of Calleja, substantia nigra, ventral tegmental area, and hippocampus[171]. As noted in an earlier section, activation of dopamine receptors by psychostimulants (e.g. cocaine) affects neural transmission of the dopaminergic system.

The involvement of the dopaminergic system on cocaine-induced behavioral effects has been extensively studied. By using selective dopamine D1 and D2 receptor antagonists, investigators have successfully assessed how dopamine receptors play a role on cocaine-induced behavioral actions. Dopamine receptor modulation affects simple behaviors such as locomotion and stereotypies, as well as complex behaviors that involve instrumental and associative conditioning paradigms. For instance, D1 and D2 receptor antagonists attenuate acute cocaine-induced locomotor activity and stereotypies (e.g., rearing, sniffing) in rats [5;170]. Conversely, D1 and D2 antagonists differentially affect chronic cocaine administration using an injection schedule that produces behavioral sensitization. Dopamine D1 antagonists (e.g., SCH 23390) block the expression of cocaine sensitization, whereas D2 antagonists (e.g., eticlopride) have no effect [110;170;183]. Interestingly, D1 and D2 antagonists have no effect on the induction of cocaine sensitization, thus suggesting that induction of cocaine sensitization is mediated by a non-dopaminergic mechanism [107;183]. The findings indicate that D1 and D2 receptors play different roles in the locomotor activity induced by acute and chronic cocaine injections.

Dopamine receptors are also involved in modulating cocaine instrumental conditioning (i.e., self-administration) and cocaine associative conditioning (i.e. conditioned place preference) paradigms. The role of D1 and D2 receptors on cocaine

self-administration is rather complex. Cocaine self-administration can be enhanced and reduced using D1 and D2 antagonists. That is, low doses of D1 and D2 antagonists enhance cocaine self-administration [73], whereas higher doses of D1 and D2 antagonists reduce self-administartion in rats [22;73]. Therefore, antagonism of D1 and D2 receptors have a biphasic role in cocaine self-administration.

Antagonism of D1 and D2 receptors also affect cocaine associative conditioning. Studies have used D1 and D2 antagonists, using the conditioned place preference paradigm, to demonstrate the involvement of dopamine receptors in cocaine-induced associative conditioning. For example, the acquisition of cocaine conditioned place preference is blocked by D1 but not D2 antagonists [5;7;27]. Moreover, low doses of the D1 antagonist SCH 23390 increased the expression of cocaine conditioned place preference, while D2 antagonists have no effect [27]. Thus it appears that D1 receptors are critically involved in the acquisition and expression of cocaine conditioned place preference, whereas the effect of D2 receptors on this paradigm is minimal, if any.

F. Cocaine-induced intracellular signal transduction:

Activation of D1 receptors causes the coupling of stimulatory G proteins (G_s or G_{olf}) to the receptors (see Figure 2). G-protein coupling stimulates adenylyl cyclase activity [80;194]. Stimulation of adenylyl cyclase causes the conversion of adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP). Enhancement in cAMP formation causes the increase of protein kinase A (PKA) activity, which then promotes the phosphorylation of various proteins, such as the cAMP response element binding protein (CREB) and the dopamine and cAMP-regulated phosphoprotein of Mr 32,000 (DARPP-32). Recent evidence suggests that CREB is a critical transcription

factor that is implicated in regulating the transcription of a number of genes involved in homeostatic adaptations, such as dynorphin [24;136]. DARPP-32, on the other hand, is a phosphoprotein that functions as a regulator of multiple signal transduction systems by the phosphorylation of its threonine 34 and 75 sites [129]. Phosphorylation of Thr-34 DARPP-32 by PKA inhibits protein phosphotase 1, which can alter the activity state of many down stream physiological effectors [61]. Meanwhile, phosphorylation of Thr-75 DARPP-32 by the cyclin dependent kinase 5 inhibits PKA activity, thus diminishing the biochemical and physiological responses to dopamine [14]. Unlike D1 receptors, activation of D2 receptors inhibits adenylyl cyclase activity by coupling to G_i proteins, inhibiting the formation of cAMP, which ultimately leads to the hyperpolarization of the cell.

Cocaine-induced changes in intracellular signaling are directly associated with behavioral effects seen after acute and chronic cocaine administration. For example, intra-accumbens infusions of a PKA activator 8-bromo-cAMP, augmented the acute locomotor activating effect of cocaine, and enhanced the sensitized response to chronic cocaine. On the contrary, intra-accumbens infusion of a PKA inhibitor R_p-CRT-cAMP, attenuated the acute locomotor activating effects of cocaine, as well as the sensitized response to cocaine after chronic administration[117]. Moreover, intra-accumbens infusions of a PKA activator Sp-cAMPS, increased cocaine self-administration and shifted the dose response curve of cocaine to the right. Intra-accumbens infusion of PKA inhibitor R_p-cAMPS, reduced cocaine self-administration and shifted the cocaine dose-response curve to the left [160]. These data indicate the importance of PKA activation in the behavioral response to cocaine.

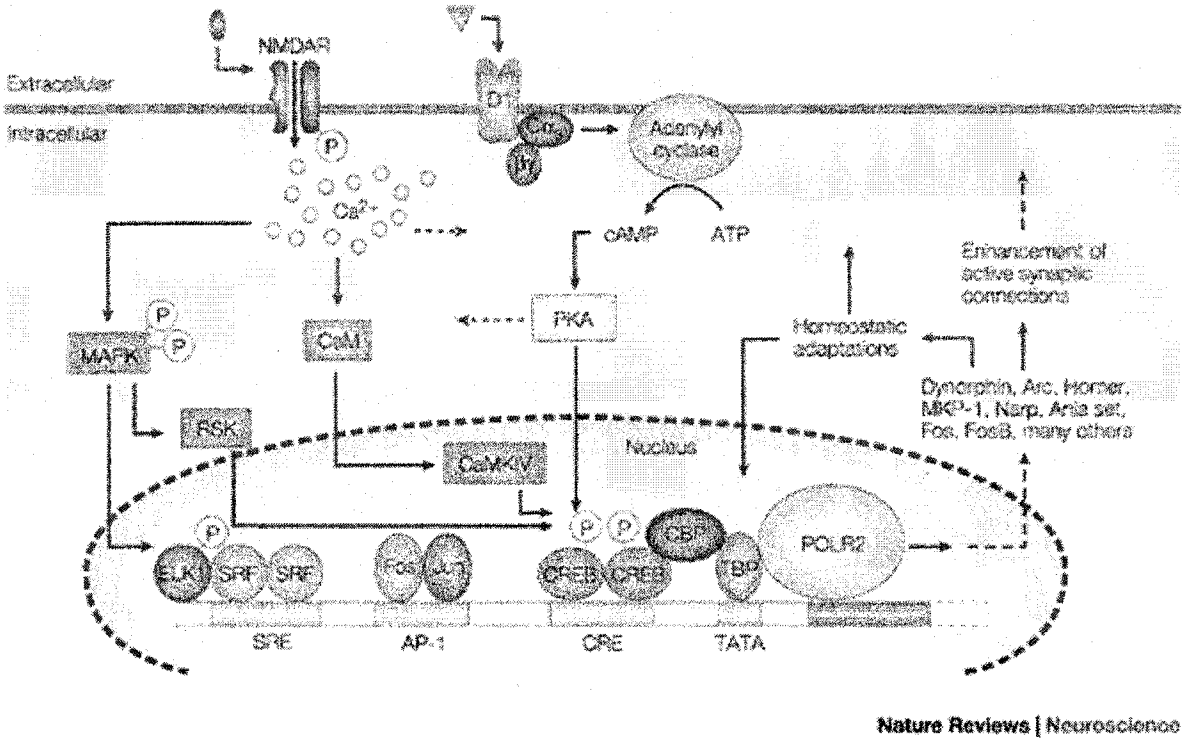

Figure 2: Intracellular transduction pathway for D1 dopamine receptors. Stimulation of the D1 receptor causes the activation of several intracellular molecules that leads to gene transcription and cellular changes. (Adopted from Hyman & Malenka 2001).

The CREB and DARPP-32 systems are likely mechanisms involved in the alteration of cocaine-induced behaviors. For instance, CREB knockout mice exhibit enhanced locomotor response to acute cocaine, cocaine sensitization and conditioned place preference response compared to CREB wild-type mice [179]. Similarly, over-expression of a dominant-negative mutant CREB in the nucleus accumbens, using inducible knockout technology, increases cocaine conditioned place preference response compared to controls. Over-expression of CREB in the nucleus accumbens decreases the rewarding effects of cocaine [24;136]. Phosphorylation of DARPP-32 also renders changes in the behavioral response to cocaine. That is, DARPP-32 knockout mice

exhibit an attenuated response to acute cocaine-induced locomotor activity, while exhibiting a more robust cocaine locomotor sensitization [52;69]. The difference in response to acute and chronic cocaine in DARPP-32 knockout mice is dependent on the phosphorylation site of the protein. Specifically, acute cocaine causes the phosphorylation of Thr-34 DARPP-32 and dephosphorylation of Thr-75 DARPP-32, which phosphorylates CREB, and thus results in the reduction in the locomotor activity [156]. On the other hand, chronic cocaine phosphorylates Thr-75 DARPP-32 and dephosphorylates Thr-34 DARPP-32, which can result in a more enhanced locomotor sensitization [156;157]. DARPP-32 is also implicated in cocaine reward, as DARPP-32 knockout mice show a reduction in cocaine conditioned place preference [189]. Altogether, activation of the cAMP second messenger alters the phosphorylation state of multiple downstream targets that can affect the behavioral response to cocaine by the way of numerous mechanisms.

G. Sex differences in the behavioral and subjective effects of cocaine:

Differences in behavioral response to cocaine have been documented between males and females. In humans, contradictory results compromise our understanding of sex dependent responses. For instance, cocaine causes a greater level of "nervousness" in women than in men [88]. Similarly, cocaine exposure also results in men achieving a faster "high" and higher peak of plasma cocaine levels, and reported more episodes of euphoria than women [102]. Conversely, there is also evidence of no sex differences in response to cocaine, as no differences in plasma cocaine levels or subjective response were detected between men and women [114]. This inconsistency may be due to the method of cocaine administration, because studies that detect sex differences utilized the

intranasal administration method, whereas the study that detected no difference utilized the I.V. method of administration. Thus, there appear to be differences in response to cocaine as a function of the route of administration.

Although direct measures for cocaine response between men and women are contradictory, some indirect measures of cocaine's effect in men and women are more consistent. For example, women cocaine addicts report a greater level of drug craving upon introduction of cocaine cues than men cocaine addicts [145]. This may suggest that women are more sensitive to cocaine than men. In fact, women begin using cocaine and enter treatment at earlier ages than men [62;115], and have a more severe cocaine use at intake than men [87]. Taking the sex difference literature in humans together, sex differences in response to cocaine are dependent on the route of administration, and indirect evidence for the subjective effects of cocaine suggests that women are more sensitive than men.

Sex differences in response to acute and chronic cocaine administration have also been measured in rodents. More specifically, an acute cocaine injection produces a greater increase in locomotor activity and rearing in females then males [19;65;158;174;175]. In addition, chronic cocaine administration produced a greater sensitization of the locomotor response in females [31;57], and sensitization was achieved with a lower dose of cocaine [138]. More recent studies have also demonstrated sex differences in the rewarding property of cocaine, as measured by the conditioned place preference paradigm [153]. Precisely, female rats exhibit a preference for the cocaine-paired compartment with lower doses of cocaine and with fewer conditioning trials, than males. On the other hand, no differences in cocaine self-administration have

been found between males and females [33;104]; however, female rats appear to self-administer saline at a greater magnitude than males during the reinstatement phase of a cocaine self-administration paradigm [104]. When considered together, a significant volume of findings suggest that females are more sensitive to the behavioral effects of cocaine than males. It is currently unclear what the underlying mechanisms are for the sex differences observed in response to cocaine. However, a possible suspect in such a mechanism might be the endogenous gonadal hormones of females. That is, gonadal hormones may act as neuromodulators to facilitate or inhibit the effects of cocaine in females.

II. Alternative mechanisms involved in the sex differences to cocaine

A. The role of estrogen and progesterone on the behavioral effects of cocaine:

The neuromodulating role of gonadal hormones on the effects of cocaine is an interesting, yet, a rather new area of study. Only a small amount of findings are currently available that demonstrates endogenous cycling estradiol and progesterone, as well as exogenous estradiol and progesterone replacement may alter an animal's response to cocaine. For instance, acute cocaine produces a greater locomotor response in female rats during the estrous stage of their cycle than the other stages of their cycle [141;161]. Moreover, acute cocaine produces an attenuated locomotor response in ovariectomized females [19]. This attenuated response is gonadal hormone dependent, because estrogen or estrogen + progesterone replacement augments the cocaine-induced locomotor response [134;140;161]. Similarly, chronic cocaine administration did not produce locomotor sensitization after 14 consecutive days of treatment in ovariectomized females

[19;133]. However, cocaine produced a robust locomotor sensitization in ovariectomized females given estradiol [71].

Estrogen and progesterone are also involved in the rewarding and reinforcing properties of cocaine. For instance, ovariectomy reduced cocaine self-administration in females, which was reversed by estradiol replacement [105;149]. Lastly, cocaine conditioned place preference was attenuated in ovariectomized females [153]. This effect was also reversed by estrogen and estrogen+progesterone treatments [152]. Interestingly, progesterone diminished the cocaine conditioned place preference compared to ovariectomized females[152]. Taken together, these findings suggest that estrogen and progesterone work as neuromodulators to alter cocaine responding in female rats.

B. The role of estrogen and progesterone on dopamine levels, release, and DAT (Pre-synaptic mechanisms):

In gonadally intact rats, no sex differences exist in basal dopamine and its metabolite levels in the NAc, whereas the levels of the dopamine metabolite DOPAC is higher in the CPu of females [51;167;187]. The release of dopamine differs between the sexes, as females release and reuptake dopamine faster than males [178]. These observations were made without consideration for the estrous cycle of females. However, changes do exist in the CPu and NAc of female rats across the different stages of the cycle. For instance, extracellular accumbal DA levels are lower and DOPAC/DA turnover rates are higher during proestrus compared to all other stages of the cycle [163]. In the CPu, extracellular DA levels are higher during proestrus and estrus compared to diestrus or in ovariectomized rats [187]. When assessing the overall content of the

dopamine in the CPu, females in estrus have greater levels of DA, while having lower DOPAC/DA turnover than males.

The effect of gonadal hormones on DA release and synthesis is further demonstrated in ovariectomized and hormone replaced rats. That is, ovariectomy reduces striatal DA release; which is reversible by acute and chronic administration of physiological doses of estradiol [41]. Similar findings exist in in vitro striatal superfusion studies, where estradiol increased basal and KCl stimulated DA release [111]. This release is likely due to an overall increase in DA synthesis as estradiol also enhanced striatal DOPAC levels. Although the precise mechanism for the increase in DA synthesis is unclear, it is possible that estrogen facilitates the phosphorylation of tyrosine hydroxylase, because striatal L-3,4-dihydroxyphenylalanine levels were increased after estrogen [132].

The role of progesterone on dopamine release is less well known due to a lack of studies addressing this question. It is known, however, that an acute physiological dose of progesterone increases striatal dopamine levels and DOPAC/DA turnover in male and female rats [40]. This effect may be due to progesterone because plasma estrogen levels were unchanged, suggesting that the effects were not due to the conversion of progesterone into estrogen.

In addition to the effects of gonadal hormones on DA release and content, recent evidence suggests the existence of sex differences in cocaine-induced changes in DA release. Specifically, acute cocaine decreases DA, DOPAC, and HVA levels in the NAc of female rats, whereas it increases DA levels in the NAc of male rats [51]. In both sexes, cocaine reduces DOPAC/DA turnover in the NAc, suggesting a reduction in DA

synthesis. The discrepancy between the sexes could be due to changes in autoreceptor-mediated regulation of DA synthesis and release in the striatum. As suggested by Becker (1999), the endocrinological profile of females contributes to the downregulation of D2 autoreceptors in the substantia nigra, which could results in enhanced DA release in the CPu.

Dopamine transporters (DAT) play an important role in the regulation of synaptic dopamine. Although evidence indicating sex differences in DAT levels are not consistent, as studies show either no difference [50] or an increase in striatal DAT levels of female rats compared to male rats [121;144]. In cycling females, DAT levels modulate at different stages and peak in the morning of proestrus when estrogen levels are high [121]. When ovariectomized, female rats possess lower levels of striatal DAT than intact females [121]. Acute and chronic estrogen administration enhances DAT in the striatum and substantia nigra pars compacta of OVX females [120;121]. Similarly, chronic administration of progesterone or combination of estrogen and progesterone enhances DAT levels in the striatum and substantia nigra pars compacta [121]. These studies demonstrate the modulatory role of gonadal hormones on dopamine uptake mechanisms.

C. The role of estrogen and progesterone on dopamine receptors (Post-synaptic mechanisms):

Sex differences in dopamine receptors could play an integral role in the way the dopamine system functions between males and females. For instance, changes in D1 receptor densities may partly explain the differences between the sexes, however, this line of evidence remains unclear, as accumbal D1 receptors densities have been shown to

either have no difference [50] or to be greater in male rats than female rats [3]. On the other hand, gonadal hormones could modulate DA receptors, as D1 receptor levels are increased during diestrous 1 and 2 when compared to OVX females. The role of gonadal hormones on D1 receptors is complicated due to conflicting findings in the literature. The difference in findings depend on a number of factors including the time during the cycle in which the rats were ovariectomized and the statistical comparisons that were utilized for the data analysis. Precisely, Levesque and Di Paolo (1990) showed that D1 receptor levels can increase if rats are ovariectomized during the proestrous or estrous, while no changes could occur if rats are ovariectomized during diestrous. Moreover, statistical comparison between groups could yield different findings in D1 receptor levels, because studies demonstrate changes in D1 levels in OVX females only when comparing to a generalized intact female group. Whereas, OVX rats do not have lower D1 levels from rats in estrous or proestrous [97], suggesting that ovariectomy only prevents the enhancement of D1 receptors during diestrous, but does not further reduce D1 levels compared to other stages of the cycle. Administration of estrogen in OVX females can result in differences in D1 receptor levels depending on the length of exposure to the hormone. That is, OVX females injected with estrogen for one week show a reduction in D1 receptors levels [169], whereas females injected with estrogen for two weeks show an enhancement in D1 receptors compared to their OVX controls [95]. Therefore, the length of exposure to estrogen could affect D1 receptor levels.

Sex differences in D2 receptors have not been reported, however, gonadal hormones modulate D2 levels in female rats. In normal cycling females, D2 receptors modulate the ratio of high-low affinity dopamine agonist binding sites starting from the

morning of proestrous to diestrous 1. Females in diestrous 2 have a much greater ratio of high to low affinity D2 binding sites than all other stages of the cycle [39]. Another report has detected reductions in affinity and D2 receptor levels in estrous females compared to males [48]. Exogenously administered gonadal hormones can also affect D2 receptor levels in male and female rats. In intact males and females, a three-day estrogen administration paradigm downregulated D2 levels in the striatum [49]. In OVX females, D2 receptor changes after estrogen treatment have reported both an increase and a decrease in D2 receptors levels. These discrepancies are likely due to differences in the type of estrogen used and the striatal subregion measured. For instance, acute and chronic administration of 17β-estradiol increases striatal D2 receptors in OVX females [39;46;59]. When the striatum is divided into lateral and medial sections, D2 enhancement is observed in the lateral, but not the medial region [46]. On the other hand, acute administration of estradiol benzoate decreases D2 receptors in the caudal striatum of OVX females [8]. These findings yield a complex relationship in the modulatory effects of estrogens on D2 receptors.

Cocaine-induced changes in DA receptors have been recently studied in intact male and female rats. Twenty four hours after acute cocaine, D1 receptors were downregulated in the CPu of males, but not females [50]. In addition, cocaine did not alter D1 receptors in the NAc of males or females. Similarly, no changes were detected in D2 receptor levels in the CPu or NAc of male or female rats. These findings suggest that compensatory mechanisms in response to cocaine may differ between male and female rats.

D. The role of estrogen and progesterone on cocaine-stimulated intracellular signal transduction:

Previous studies have shown that the neuromodulatory effects of estrogen and progesterone alter cocaine responding in various behavioral paradigms. Although, estrogen and progesterone work as neuromodulators, their underlying mechanisms are not well understood. For instance, it is unclear if their interaction with cocaine is genomic or non-genomic. It is also unclear whether estrogen and progesterone affect signal transduction cascades responsible for cocaine's behavioral effects. It is known, however, that both estrogen and progesterone modulate signal transduction cascades in the brain. For example, estradiol treatment alters mRNA levels of dopamine receptors in the brain. A recent finding suggests that estradiol replacement robustly increases D1 mRNA levels in the hypothalamus [193]. Furthermore, D2 receptor mRNA was upregulated in the midbrain, while D3 receptor mRNA was upregulated in the ventral tegmental area, but downregulated in the midbrain [193]. Moreover, ovariectomy decreases dopamine D1-sensitive adenylate cyclase activity in the striatum of female rats [90]; whereas, treatment of estradiol to primary striatal cultures enhances dopamine activated D1-sensitive adenylate cyclase activity and suppresses dopamine inhibited D2-sensitive adenylate cyclase activity [108]. Estradiol-induced changes in signal transduction mechanism are further demonstrated in the hypothalamus and the pituitary gland, regions that contain numerous transmitters, such as dopamine, dynorphin and enkephalin. Estradiol treatment increases phosphorylated CREB immunoreactivity in the anteroventral periventricular nucleus of the hypothalamus [63], whereas it decreases phosphorylated CREB levels in the pituitary [42]. Estradiol has been implicated in the

phosphorylation of DARPP-32 in various nuclei of the female hypothalamus. Recent findings demonstrate that estradiol enhanced Thr-34 DARPP-32 phosphorylation in the medial preoptic nucleus, bed nucleus of the stria terminalis, paraventricular nucleus, and the ventromedial nucleus of the hypothalamus [4]. Taken together, these findings demonstrate that estradiol is involved in modulating dopaminergic intracellular signal transduction mechanisms.

Studies assessing the role of progesterone on cocaine-induced intracellular signaling are limited. Recent findings suggest that progesterone alone does not alter cAMP levels during basal conditions [1]. However, progesterone enhanced forskolin-induced cAMP levels in the parietal and occipital cortex, while progesterone inhibited forskolin-induced cAMP levels in the frontal cortex [1]. Progesterone's intracellular signal transduction pathway is very similar to that of dopamine. Both utilize the cAMP system to promote gene expression [38;162]. For instance, activation of PKA using 8-bromo-cAMP (a PKA activator) mimicked progesterone and dopamine receptor mediated gene transcription [38;117]. It has been demonstrate that progesterone and dopamine enhance cAMP and PKA activation and increase Thr-34 DARPP-32 phosphorylation in the hypothalamus [106]. The dopamine-induced increase in signal transduction is reversed by the D1 antagonist SCH 23390, suggesting that the increase was D1 receptor mediated. Conversely, dissociation between the signal transduction of dopamine and progesterone seems to exist as progesterone-induced increase in signal transduction is not affected by D1 antagonists [106]. The above-mentioned studies indicate that estrogen and progesterone have an integral neuromodulatory effect in dopamine signaling. However, there is a lack of evidence linking the involvement of estrogen and

progesterone in the signal transduction mechanisms involved in cocaine's actions in brain regions such as the nucleus accumbens and the caudate putamen.

III. Significance of work

As noted in an earlier section, better understanding the underlying mechanism of the difference in cocaine's actions between males and females is critical. It is known that over 30% of cocaine abusers are females and that females experience different subjective effects to cocaine than males. The animal literature has shown that sex differences due to cocaine are apparent in various behavioral paradigms. However, no studies have addressed the cellular mechanisms that may control the sex differences in behavioral responses to cocaine. Understanding the cellular mechanisms by which these sex differences are produced may help the scientific community develop more effective therapeutic methods.

Our working hypothesis is divided into three levels. First, it is predicted that there are sex differences in the functioning of D1 and D2 receptors. That is, females will respond differently to D1 and D2 antagonists in the cocaine conditioned place preference paradigm. Second, it is predicted that cocaine-induced locomotor activity and behavioral sensitization will differ between male and female rats. Lastly, the differences in the cocaine-induced locomotor activity and behavioral sensitization are due to differences in the functioning of the signal transduction mechanisms. Detecting sex differences at these three levels will in turn increase our understanding of how cocaine's actions differ between males and females.

A. AIMS:

Specific Aim One: To determine if sex differences exist in the functioning of dopamine receptors in a model of cocaine reward and locomotion.

1A: To determine whether sex differences exist in blocking the acquisition of cocaine conditioned place preference and cocaine-induced locomotion using a D1 receptor antagonist.

1B: To determine whether sex differences exist in blocking the acquisition of cocaine conditioned place preference and cocaine-induced locomotion using a D2 receptor antagonist.

Specific Aim Two: To determine whether sex differences exist in the time course for cocaine-induced activation of intracellular signal transduction mechanisms.

2A: To determine if there are sex differences in the time course of the catalytic subunit of PKA.

2B: To determine if there are sex differences in the time course of CREB phosphorylation.

Specific Aim Three: To determine whether cocaine-induced dopamine signal transduction is altered after chronic cocaine.

3A: To determine if there are sex differences in behavioral sensitization to cocaine.

3B: To determine if the sex differences in the sensitization are due to differential phosphorylation of CREB in the caudate putamen and nucleus accumbens.

Chapter 2: The role of D1 and D2 receptors in the cocaine conditioned place preference of male and female rats.

I. Introduction

Cocaine induces a greater enhancement of behavioral responses in female rats than in male rats. When administered acute or chronic cocaine, female rats exhibit a more robust locomotor activity and behavioral sensitization than do male rats [31;57;65;158;161;174;175]. Sex differences also exist in the reinforcing and rewarding properties of cocaine. For example, female rats demonstrate a more rapid acquisition to cocaine self-administration, reach a higher breaking point in a progressive-ratio schedule, and have a greater response during the reinstatement phase than do male rats [33;103;104;148]. Sex differences also exist in the conditioned rewarding effects of cocaine using a conditioned place preference (CPP) paradigm; female rats developed CPP using lower doses of cocaine and with fewer conditioning days than did male rats [153]. Although there is increasing evidence that suggests the existence of sex differences in behavioral responses to cocaine, the underlying neuronal mechanisms for these differences are not clear.

The dopaminergic system is important in regulating the rewarding properties of psychostimulants [10;21;185]. In male rats, D_1 but not D_2 receptors are involved in the acquisition of cocaine conditioned place preference [5;7;27;139]. Female rats have lower D_1 receptor densities in the nucleus accumbens, and greater dopamine release, reuptake and dopamine transporter density in the striatum [3;12;144;178]. We predict that the observed sex differences in the conditioned rewarding effects of cocaine are in part

mediated through differential activation of the D_1 and D_2 receptors. The aim of the current study is to test this postulate.

II. Method

Subjects:

Eight-week-old male and female Fischer rats (Charles River, Raleigh, NC, USA) were individually housed in standard cages with free access to food and water and maintained on a 12-hour light/dark cycle (lights on at 8:00 am). All rats were weighed and handled for five days prior to experimental manipulations. Animals were randomly assigned to experimental groups (n = 9-11 rats/group). Consideration of the estrous cycle was purposely excluded from the present study because repeated vaginal lavage produces place preference and reduces cocaine-induced activity [177]. These effects may disrupt the development of cocaine CPP, thereby rendering findings difficult to compare with male rats. After testing, animals were decapitated following a 20 s exposure to CO_2. Animal care was in accordance with the Guide for the Care and Use of Laboratory Animals (National Institute of Health publication 85-23, Bethesda, MD, USA) and approved by the Institutional Animal Care and Use Committee at Hunter College.

Drugs:

Cocaine hydrochloride and S(-)-eticlopride were purchased from Sigma (St. Louis, MO, USA). SCH 23390 hydrochloride was purchased from Tocris Cookson (Ellisville, MO, USA). All drugs were dissolved daily in saline (0.9%) and administered intra-peritoneally (i.p.) in a volume of 1 mL/kg.

CPP apparatus:

Place preference cages were purchased from Med Associates (Georgia, VT, USA) and consist of a rectangular box with three distinct chambers. The center neutral gray chamber was 12 cm long, with a smooth PVC floor. The two conditioning chambers were 28 cm long and had different visual and tactile cues. One chamber was black with a stainless steel grid floor, and the other was white with stainless steel mesh floor. The chambers were separated by computer-automated guillotine doors to allow access to all chambers.

CPP Procedure:

A 4-day CPP paradigm was used, similar to the one previously described by Russo et al. (2003). In brief, rats were placed into a neutral gray chamber for a 5-min acclimation period during the preconditioning phase, and then allowed free access to all chambers for 15 min. Eight cohorts of rats were then randomly assigned to either a cocaine/saline conditioning group or a saline/saline control group. Cocaine pairings were evenly distributed between the black and the white chambers, rendering an unbiased paradigm. On conditioning days 1 and 3, rats were pretreated in the home cage with SCH 23390 (0.10, 0.25, or 0.50 mg/kg), eticlopride (0.05, 0.10, or 0.25 mg/kg), or vehicle (saline). Fifteen minutes after the pretreatment injection, rats received saline or cocaine (5 mg/kg for females and 20 mg/kg for males) and were immediately placed in one of the conditioning chambers for 30 min. These doses have previously been shown to represent the optimal doses needed to produce CPP in each sexe [153]. On alternate days, rats were injected with saline 15 min after the antagonist injection and were

immediately placed in the opposite chamber for 30 min. Control rats received a similar antagonist pretreatment and saline on days 1-4 of conditioning in both chambers. On the testing day, rats were placed into the neutral gray chamber for a 5-min acclimation period and then allowed 15 min free access to all three chambers in a drug-free state. The time spent was recorded using a computerized photo-beam system run with MED-PC software.

Statistics:

Statistically significant differences of time spent in each chamber were analyzed using planned paired sample t-tests within each group. CPP scores (time spent in cocaine-paired chamber minus time spent in saline-paired chamber) were analyzed using a one-way ANOVA in female and male rats pretreated with vehicle antagonist and conditioned with cocaine. Significance in all cases was considered at the 0.05 probability level ($p < 0.05$).

III. Results

Throughout the study, control rats (saline/saline) did not differ in the time spent in either chamber, confirming the unbiased nature of the procedure (Table 1). In addition, rats injected with SCH 23390 or eticlopride and saline did not exhibit any preference for either of the chambers. There was no significant difference in CPP scores in female and male rats pretreated with vehicle antagonist and conditioned with cocaine between the two experiments $F(3,39) = 0.7674$, $p > 0.05$.

As shown in Figure 3, both female and male rats pretreated with SCH 23390 vehicle and cocaine demonstrated CPP [Females: $t(10) = 3.067$, $p < 0.05$; Males: $t(10) = 4.185$, $p < 0.05$]. In female rats, 0.10 and 0.25 mg/kg SCH 23390 blocked cocaine CPP, whereas the 0.50 mg/kg dose of SCH 23390 did not block cocaine CPP [0.10 mg/kg: $t(10) = 0.165$, $p > 0.5$; 0.25 mg/kg: $t(10) = 2.061$, $p > 0.05$; 0.50 mg/kg: $t(10) = 2.860$, $p < 0.05$]. In male rats, all SCH 23390 doses blocked cocaine CPP [0.10 mg/kg: $t(10) = 1.923$, $p > 0.05$; 0.25 mg/kg: $t(10) = 1.678$, $p > 0.05$; and 0.50 mg/kg: $t(10) = 0.079$, $p > 0.05$]. As shown in Figure 4, female and male rats pretreated with eticlopride vehicle and cocaine demonstrated CPP [Females: $t(8) = 5.190$, $p < 0.001$; Males: $t(8) = 2.514$, $p < 0.05$]. However, none of the eticlopride doses affected cocaine CPP in either female or male rats [Females: 0.05 mg/kg: $t(8) = 2.795$, $p < 0.02$; 0.10 mg/kg: $t(8) = 5.916$, $p < 0.001$; 0.25 mg/kg: $t(8) = 3.140$, $p < 0.01$; Males: 0.05 mg/kg: $t(8) = 4.540$, $p < 0.001$; 0.10 mg/kg: $t(8) = 2.322$, $p < 0.05$; 0.25 mg/kg: $t(8) = 3.045$, $p < 0.01$].

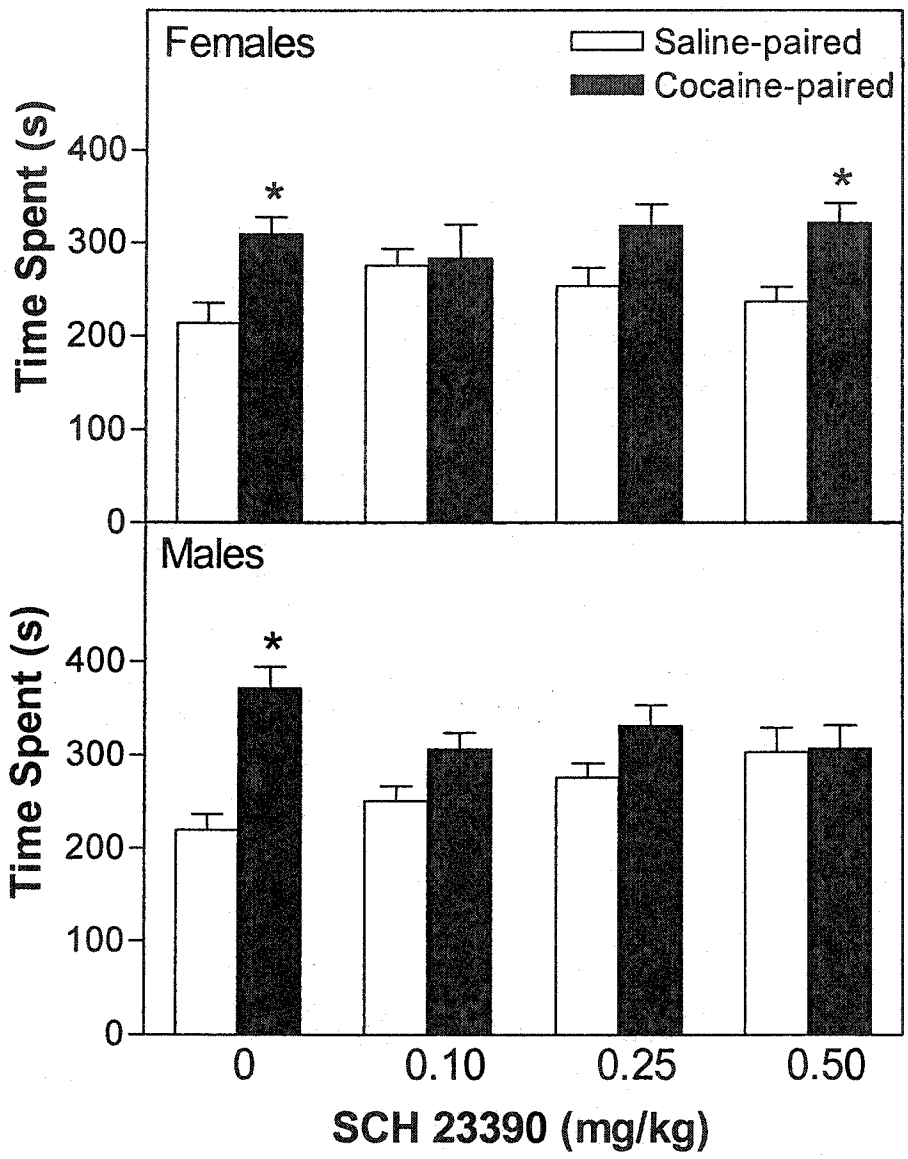

Figure 3: The effects of SCH 23390 on cocaine CPP of female and male rats. Data represents the time spent in seconds (±S.E.M.) in saline-paired (white bars) and cocaine-paired (black bars) compartments on the test day. * Indicates significant difference ($p < 0.05$) between saline- and cocaine-paired compartments within antagonist dose.

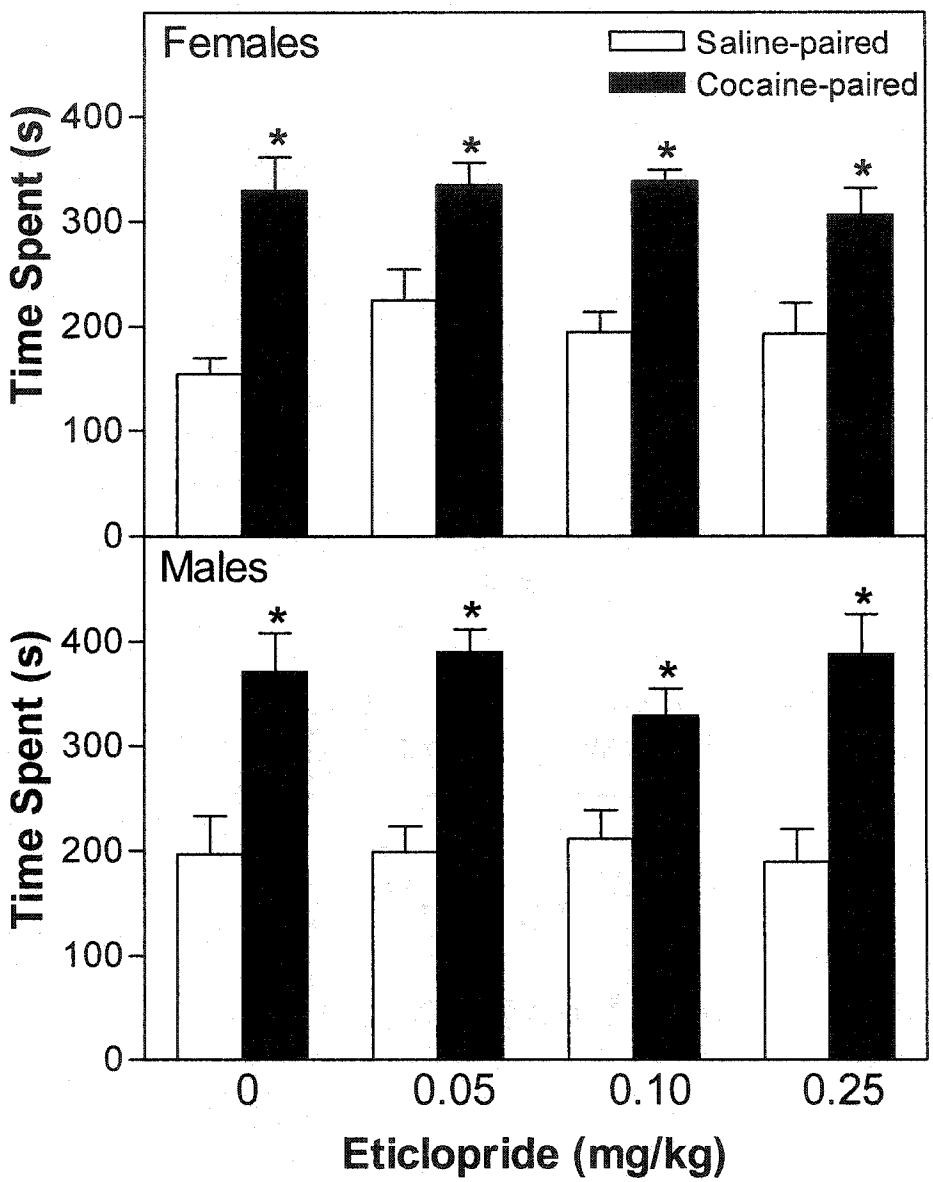

Figure 4: The effects of eticlopride on cocaine CPP of female and male rats. Data represents the time spent in seconds (±S.E.M.) in saline-paired (white bars) and cocaine-paired (black bars) compartments on the test day. * Indicates significant difference ($p < 0.05$) between saline- and cocaine-paired compartments within antagonist dose.

Table1: Time spent in each CPP compartment after vehicle, SCH 23390, or eticlopride in saline treated rats.

	Female		Male	
Chambers	White	Black	White	Black
Antagonists (mg/kg)				
SCH 23390				
0	250.1 ± 25.1	281.1 ± 23.5	283.3 ± 20.5	249.0 ± 20.4
0.10	256.8 ± 16.4	255.4 ± 23.4	258.1 ± 24.3	286.8 ± 15.5
0.25	284.5 ± 17.4	268.7 ± 12.4	276.2 ± 22.5	315.7 ± 18.9
0.50	288.6 ± 31.0	303.7 ± 29.3	328.0 ± 22.5	267.8 ± 23.1
Eticlopride				
0	232.9 ± 19.3	204.9 ± 15.3	259.8 ± 28.8	271.0 ± 45.2
0.05	229.4 ± 18.5	278.4 ± 22.8	283.3 ± 32.5	258.3 ± 29.1
0.10	213.0 ± 21.3	275.7 ± 16.7	239.1 ± 29.1	328.3 ± 40.5
0.25	220.2 ± 19.3	274.7 ± 17.3	234.1 ± 21.7	273.8 ± 30.5

Rats pretreated with SCH 23390 or eticlopride did not exhibit significant differences in time spent between the two compartments. Data presented as mean time spent in seconds (± S.E.M.).

IV. Discussion

Similar to previous studies in male rats, the present study demonstrates that D_1, but not D_2, receptors are involved in the acquisition of cocaine CPP in female rats. Moreover, sex differences were observed in the antagonistic properties of D_1 receptors in cocaine CPP. These findings are consistent with Schindler and Carmona (2002), which demonstrated that sex differences exist in the antagonism of D_1 but not D_2 receptors for cocaine-induced locomotor activity. Taken together, our results suggest that differences in D_1 receptor activity in male and female rats may be involved in the development of learned associations between environmental cues and cocaine reward.

Three interrelated postulates may underlie the inability of the high dose of SCH 23390 to block cocaine CPP in female rats. First, in male rats, it has been demonstrated that intra-accumbal injection of a high dose of SCH 23390 occupied fewer D_1 receptor sites than a lower dose of SCH 23390 [125]. Due to differences in dopamine tone and D_1 receptor densities in mesolimbic areas [3;11;37;178], the dose-dependent binding characteristics of SCH 23390 for D_1 receptors may have been shifted in female rats, and thus attenuated the potency or efficacy of the high dose of SCH 23390 in blocking cocaine CPP. Second, high doses of SCH 23390 have affinity for the 5-HT_{2A} and 5-HT_{2C} receptors in male rats [15;32;116]. Female rats have higher serotonin 5-HT_{2A} receptor mRNA levels in brain regions involved in learning and memory (e.g. hippocampus) [192]. On the other hand, no sex differences have been demonstrated in 5-HT_{2C} receptors; it is unknown whether the 5-HT_{2C} receptors are involved in the observed effects of SCH 23390. Third, sex differences in the overall serotonin tone and activity have been demonstrated. For instance, female rats have higher basal and cocaine-induced

serotonin levels in the nucleus accumbens and the striatum than do male rats [25;51]. Differences in the firing rate of serotonin neurons of the raphe nucleus have also been reported, with male rats having higher spontaneous firing rates than female rats [84]. Due to the involvement of serotonin in cocaine CPP [2;128], serotonergic activation (either by activation of its receptors and/or through interactions with the dopamine system) may be more profound in female rats, which could explain the inability of the high dose of SCH 23390 to block cocaine CPP in female rats. These postulates are currently under investigation in our group.

Learning and memory processes serve as the basis for the acquisition of conditioned place preference to cocaine. Sex differences in the neuronal mechanisms controlling learning and memory have been reported [18;55;152]. In addition, antagonism of D_1 and D_2 receptors produce sexually dimorphic responses in classical and instrumental learning paradigms [118;137;155]. Gonadal hormone effects in dopamine and serotonin activity are involved in learning and memory processes [101;112], and these hormones also regulate the activation of D_1 and D_2 receptors [17;47;92;96] . Although the present study does not address the effect of gonadal hormones on the regulation of D_1 and D_2 receptors on cocaine CPP, recent findings from our laboratory have shown that when administered alone, estrogen and progesterone enhance and inhibit the development of cocaine CPP, respectively. However, the combination of the two hormones enhances the development of cocaine CPP [152]. Thus, it is possible that gonadal hormones may modulate the effects of D1 antagonists on the acquisition of cocaine CPP. The extent to which the hypothalamic-pituitary-gonadal axis controls sex differences in the ability to associate and recall the rewarding effects of cocaine remains

to be elucidated. Further determination of the interactions between these systems will dramatically improve our current understanding of the sex differences in cocaine abuse.

Chapter 3: Sex differences in cocaine-induced behaviors and signal transduction mechanism.

I. Introduction

Cocaine is a powerful psychostimulant that has a high abuse potential. It is estimated that from the 1.3 million American cocaine abusers approximately 33% are women [166]. The disproportion in men and women cocaine abusers has been attributed to the likelihood of men having a greater initial opportunity to abuse drugs. However, if given the opportunity, women initiate cocaine use sooner, take less to become addicted to cocaine, and have more emergency room visits following cocaine use [43;62;98;181]. Similar to humans, female rats exhibit a greater enhancement and longevity to cocaine-induced locomotor and rearing activity, as well as behavioral sensitization to cocaine when compared to male rats [30;51;161;174;176]. Female rats also show a more rapid acquisition of cocaine self-administration and conditioned place preference than male rats [103;153]. However, to date, few studies have addressed what cellular mechanisms may underlie these striking sex differences in the behavioral response to cocaine.

The psychostimulant actions of cocaine are produced, in part, by an enhancement in the activity of the dopamine (DA) system in the striatum [74;165]. In brief, the increase of DA in the synaptic cleft after cocaine administration results in the stimulation of D1 receptors and the concomitant activation of protein kinase A (PKA). Active PKA phosphorylates the transcription factor cAMP response element binding protein (CREB). However, few studies have assessed the role of CREB in acute cocaine-induced behavioral activation. It has been shown that an acute cocaine injection produce a similar

increase in locomotor responding in CREB$^{\alpha\Delta}$ mutant mice and their wild-type counterparts [179]. On the other hand, cocaine does increase phosphorylated CREB (P-CREB) levels in the NAc and CPu of mice [79;180]; which suggests that cocaine-induced elevation of P-CREB may have an effect in motoric behaviors.

To date, great advances have been made in understanding the cellular mechanisms involved in cocaine-induced responses in male rats, whereas, it is yet to be known whether similar mechanisms are involved the cocaine-induced responses of female rats. Due to sex differences in the behavioral responses to cocaine, the findings from the male rat literature may not directly apply to that of female rats. It is not clear if the sex differences exist in the functioning of the cAMP pathway, and whether the signal transduction mechanisms respond differently to acute cocaine in the female NAc and CPu. The purpose of the present study was to assess whether the sex differences in acute cocaine-induced ambulatory and rearing activites are due to changes in intracellular signal transduction mechanisms.

II. Method

Subjects:

60-day-old male and female Fischer rats (Charles River, Raleigh, NC) were individually housed in Plexiglas chambers (20 × 20 × 41 cm) layered with beta chips. Rats were given free access to standard lab chow and water and were maintained on a 12-hour light/dark cycle (lights on at 8:00 a.m.). All rats were weighed, handled, and injected with saline (i.p.) for 5 consecutive days prior to testing. Animals were randomly assigned to experimental groups (n= 7-8 rats/group). Animal care and use was in

accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, Bethesda, MD) and approved by the Institutional Animal Care and Use Committee of Hunter College.

Drug and antibodies:

Cocaine hydrochloride was purchased from Sigma chemical Co. (St. Louis, MO). Cocaine solutions were prepared daily by dissolution in physiological saline (0.9%) and injected intra-peritoneally (i.p.) at a volume of 1 ml/kg. The primary antibody for PKA_C was purchased from Becton Dickinson Transduction Laboratories (Lexington, KY), antibody for P-CREB was purchased from Cell Signaling Technologies (Beverly, MA), and antibody for α-tubulin was purchased from Santa Cruz Technologies (Santa Cruz, CA). The appropriate secondary antibodies were purchased from Amersham Pharmacia (Piscataway, NJ).

Cocaine administration paradigm and behavioral measurements:

On the day of testing, rats were given an injection of saline or cocaine (30 mg/kg) and sacrificed 5, 15, 30, 45, or 90 min later. All behavioral measurements were conducted in the rat home cage. Behavioral activity was obtained from rats in the 90 min groups. Ambulatory and rearing activities were measured using a two frame automated Photobeam Activity System (San Diego Instruments, San Diego, CA). Ambulatory activity was determined by total counts of two consecutive photobeam interruptions in the lower frame. Rearing activity was represented as total counts of vertical motions detected by the upper frame.

Protein measurements:

After decapitation (following a brief 20 s exposure to CO_2), rat brains were removed, flash frozen in 2-methylbutane (-40° C), and stored at -80° C until used. The CPu and NAc were dissected from coronal sections (1mm thick) and homogenized using a Polytron handheld homogenizer (Kinematica, Luzern, Switzerland) in a buffer containing HEPES 7.9 (20mM), KCl (10mM), EDTA (1mM), NP40 (0.2%), Glycerol (10%), NaCl (200mM), Pepstatin, Leupeptin, DTT (1M), Aprotenin, PMSF (100mM), NaF (50mM), & Na_3VO_4 (1mM). Total protein content was determined using a Bradford kit from Bio-Rad Laboratories (Hercules, CA). Protein samples (10 μg for PKA and 25 μg for P-CREB) were boiled in Lammeli buffer containing 1% β-mercaptoethanol, centrifuged, and loaded onto 10% SDS-PAGE. Gels were then electrophoresed, transferred to nitrocellulose membranes, and blocked for 30 min with 5% non-fat dry milk in tris-buffer-saline-tween (TBST) at room temperature. Membranes were probed overnight at 4°C with PKA_C (1:1000) or P-CREB (1:500) antibodies. After three washes with TBST, membranes were incubated with their appropriate secondary antibody (1:1000) for 60 min at room temperature followed by three more washes with TBST. Antibody binding was detected using an enhanced chemiluminescence kit (ECL; Amersham Pharmacia, Piscataway, NJ). Intensity of protein bands was quantified with a computer densitometer and Image Quant Program (Molecular Dynamics). For normalization of protein levels, all membranes were re-probed with α-tubulin antibody (1:1000).

Statistical analysis of data:

Ambulations and rearing counts were analyzed using three-way repeated measures analysis of variance (ANOVAs) for the variables sex (male vs. female), drug (saline or cocaine), and time (18 five-min time blocks). In addition, one-way ANOVAs were used to determine differences within each time block. For Post hoc analysis, Tukey tests were conducted when appropriate. For analyzing protein levels, unpaired-samples t-tests were performed for pair-wise comparisons. Western blot data are presented as a ratio of PKA_C or P-CREB over α-tubulin as arbitrary densitometric units. Determination of statistically significant differences was considered at the 0.05 probability level ($p < 0.05$).

III. Results

Sex difference in cocaine-induced ambulation and rearing activities: Overall, cocaine enhanced the ambulatory and rearing activity when compared to saline [Drug main effect, Ambulations: $F(1,26) = 39.530$, $p < 0.001$; Rearing: $F(1,26) = 86.376$, $p < 0.001$; Figure 5]. Female rats exhibited a longer lasting ambulatory response to cocaine than male rats [Drug × Sex × Time interaction, $F(17,442) = 2.961$, $p < 0.05$; Figure 5A]; female rats had greater number of ambulations during the final 50 minutes of the testing session [time block 11-18; block 11: $F(3,26) = 23.42$, $p < 0.0001$; block 12: $F = 30.05$, $p < 0.001$; block 13: $F = 18.40$, $p < 0.01$; block 14: $F = 15.33$, $p < 0.0001$; block 15: $F = 37.95$, $p < 0.0001$; block 16: $F = 24.17$, $p < 0.0001$; block 17: $F = 15.56$, $p < 0.0001$; block 18: $F = 31.56$, $p < 0.0001$]. Similarly, female rats exhibited higher rearing counts after cocaine administration than male rats [Drug × Sex × Time interaction, $F(17,442) =$

3.082, p < 0.05; Figure 5B]; where cocaine-treated female rats had greater number of rearing counts from minutes 40-80 than male rats [time blocks 8-16; block 8: F (3,26) = 12.87, p < 0.0001; block 9: F = 16.38, p < 0.0001; block 10: F = 35.97, p < 0.0001; block 11: F = 37.32, p < 0.0001; block 12: F = 63.67, p < 0.0001; block 13: F = 39.47, p < 0.0001; block 14: F = 26.53, p < 0.0001; block 15: F = 75.61, p < 0.0001; block 16: F = 28.78, p < 0.0001].

Sex differences in signal transduction protein levels:

In the NAc of female rats, basal levels of PKA_C but not P-CREB were higher than that of male rats [t (6) = 2.840, p < 0.03; Figure 6A&B]. In the CPu, basal levels of PKA and P-CREB were similar between male and female rats (Figure 7A&B). After cocaine administration, P-CREB levels increased in the NAc of both male and female rats (Figure 8A&B). However in male rats, P-CREB levels were increased from 5 to 30 min (5 min: t (6) 3.447, p < 0.02; 15 min: t (6) = 2.478, p < 0.05; 30 min: t (6) = 2.826, p < 0.03), whereas, in female rats, P-CREB levels were only enhanced for 5 min [t (6) = 2.485, p < 0.05]. Cocaine administration did not alter PKA_C levels in the NAc of male and female rats (Figure 9). Moreover, cocaine had no effect on PKA or P-CREB levels in the CPu of male and female rats after cocaine administration (Table 2).

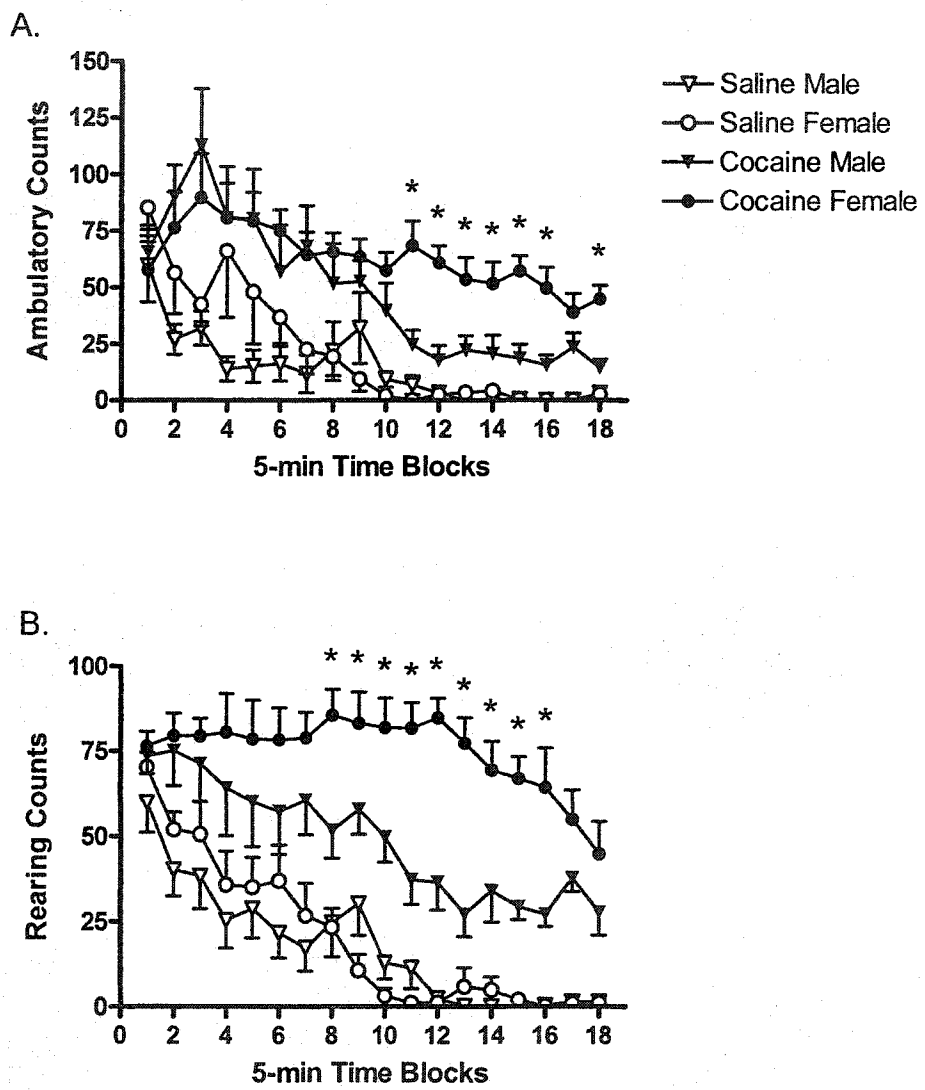

Figure 5:. Mean counts (± SEM) in male and female rats for Ambulations (A) and rearing (B) after an acute injection of cocaine (30 mg/kg). Behaviors were measured for 90 min and represented in 5-min time blocks. * Represents a significant difference between male and female cocaine treated rats at a given time block.

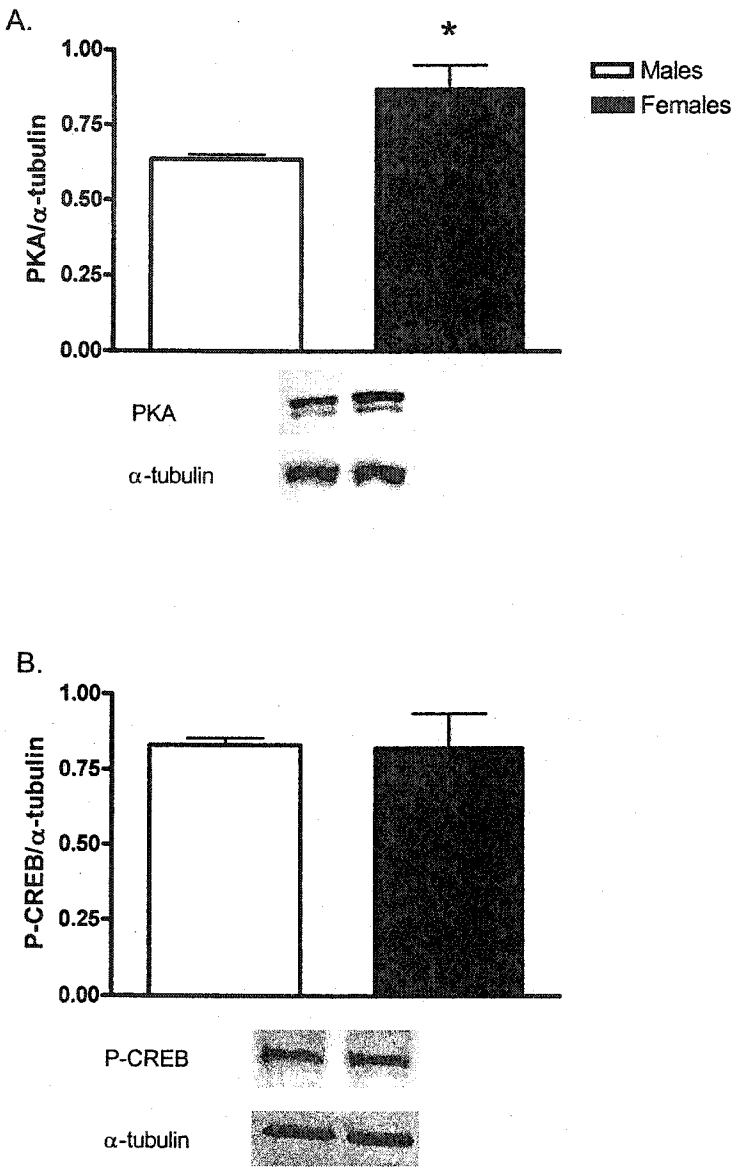

Figure 6: Mean densitometric units (± S.E.M) of basal PKA_C (A) or P-CREB (B) levels in the NAc of male and female rats. Protein levels are normalized by α-tubulin.

* Represents a significant difference between male and female rats.

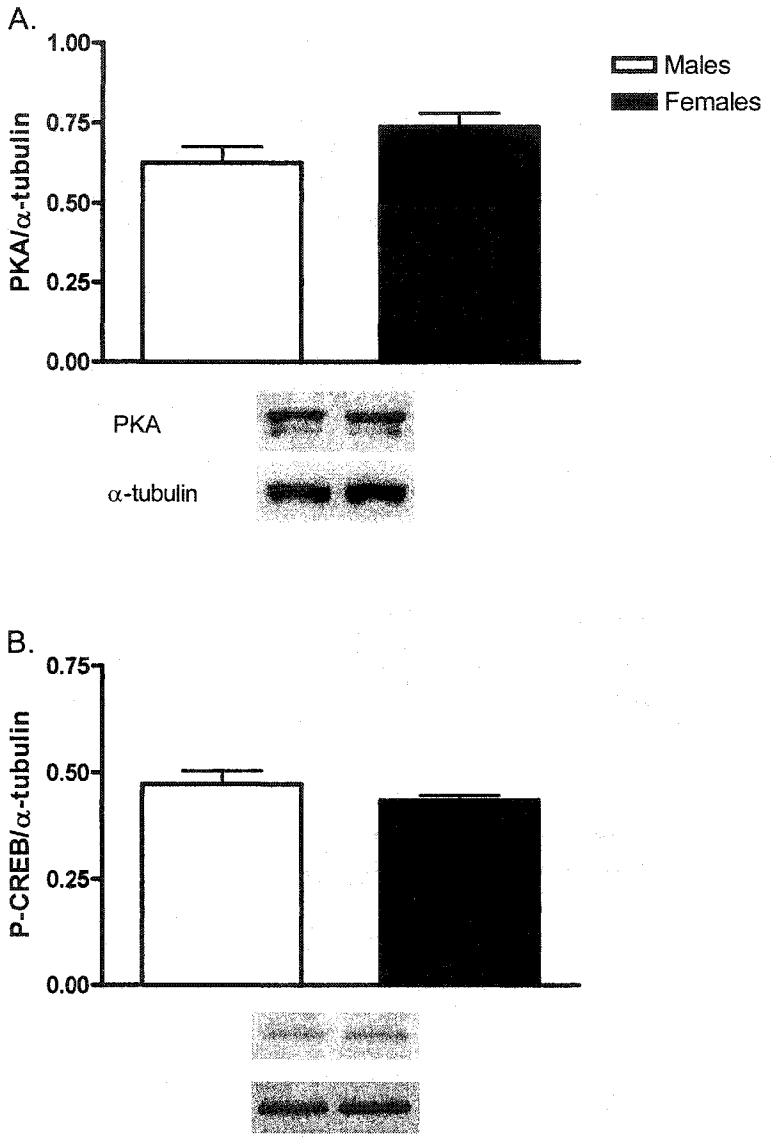

Figure 7: Mean densitometric units (± S.E.M) of basal PKA_C (A) or P-CREB (B) levels in the CPu of male and female rats. Protein levels are normalized by α-tubulin.

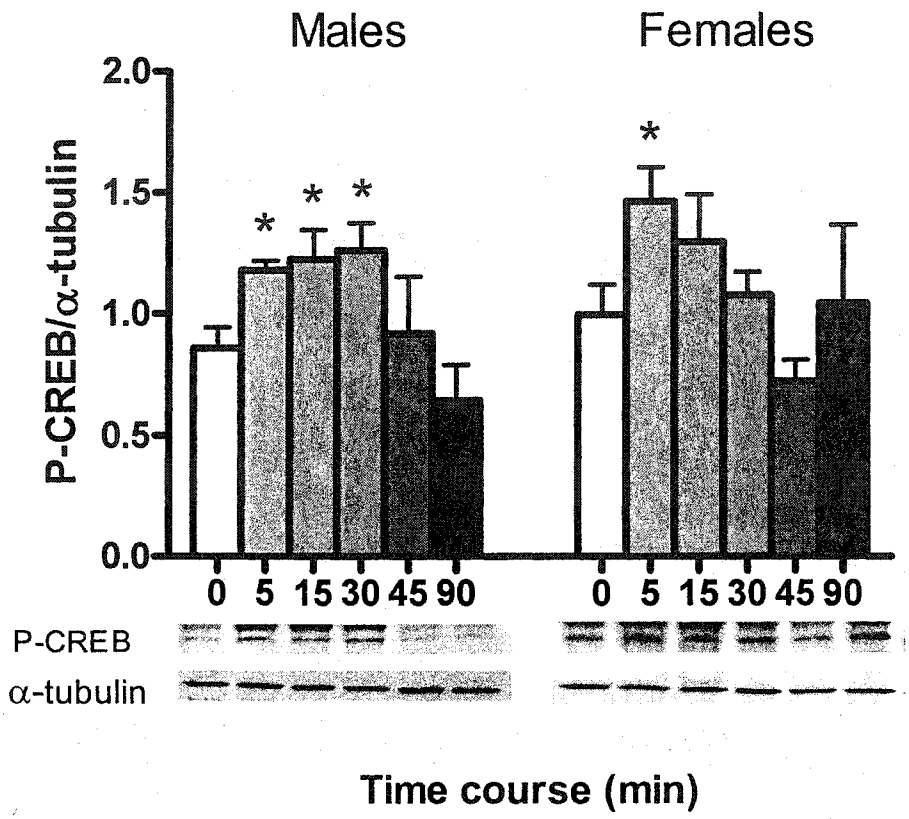

Figure 8: Mean densitometric units (± S.E.M) of P-CREB levels in the NAc of male and female rats after an acute injection of cocaine (30 mg/kg) and sacrificed 5, 15, 30, 45, and 90 min after cocaine administration. Protein levels are normalized by α-tubulin.

* Represents a significant difference between a given time point and 0 min of the same sex rat.

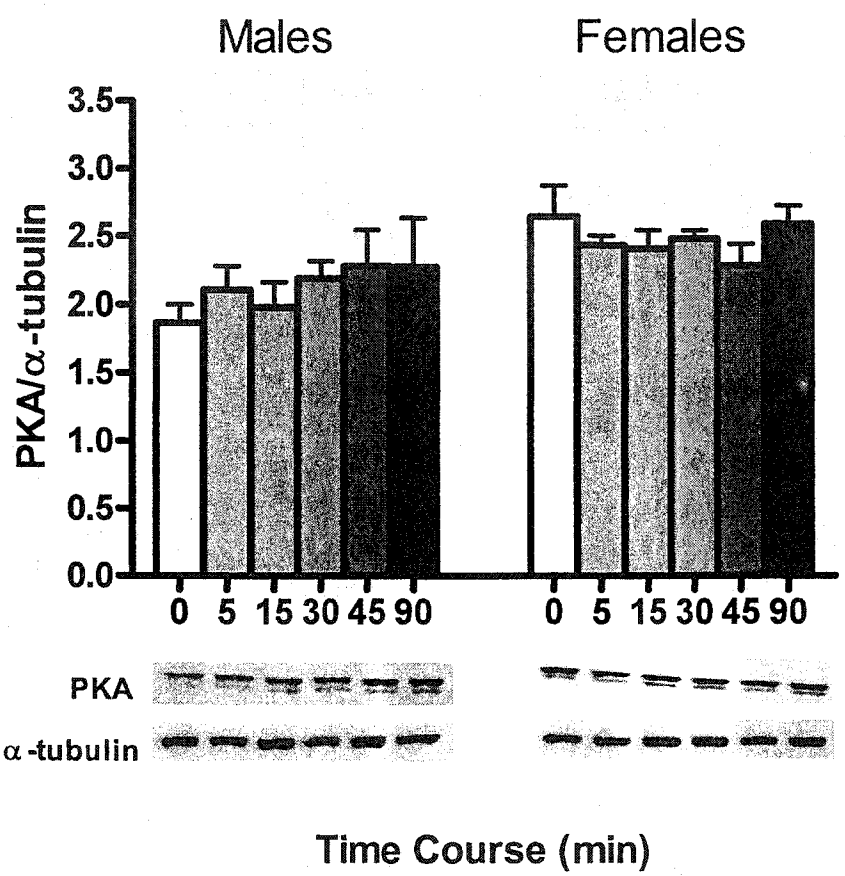

Figure 9: Mean densitometric units (± S.E.M) of PKA_C levels in the NAc of male and female rats after an acute injection of cocaine (30 mg/kg) and sacrificed 5, 15, 30, 45, and 90 min after cocaine administration. Protein levels are normalized by α-tubulin.

Table 2: The effects of acute cocaine administration on PKA_C and P-CREB levels in the CPu of male and female rats.

Sex	Protein	Time Course (min) 0	5	15	30	45	90
Males							
	PKA_C	3.38±0.36	3.56±0.32	3.07±0.30	3.08±0.18	2.68±0.57	3.49±0.19
	P-CREB	1.44±0.19	1.16±0.09	1.20±0.15	1.27±0.10	1.20±0.08	1.35±0.28
Females							
	PKA_C	3.46±0.21	3.70±0.23	3.33±0.30	3.85±0.21	3.80±0.29	3.99±0.19
	P-CREB	1.36±0.16	1.40±0.21	1.22±0.15	1.24±0.16	1.18±0.14	1.16±0.16

Time course of PKA_C and P-CREB levels in the CPu after acute cocaine (30 mg/kg) in male and female rats.
Cocaine had no effect on PKA_C or P-CREB levels in the CPu of male and female rats.
Data is represented as mean densitometric units (± S.E.M).

IV. Discussion

Similar to previous reports, the current findings demonstrate that cocaine produces a longer lasting ambulatory and rearing response in female rats than in male rats [30;51;176]. However, we show for the first time that the sex differences in cocaine-induced behaviors may be mediated through intrinsic sexual dimorphisms in cellular mechanisms known to mediate the responses to cocaine. Female rats had higher basal levels of PKA in the NAc than male rats, which suggest an inherent difference in the way PKA-mediated signal transduction mechanisms function and respond to cocaine. In male rats, pharmacological activation of PKA enhances cocaine-induced behavioral responding [117;159]. Due to the greater basal PKA levels in female rats, downstream signal transduction protein involved in cocaine's actions may be different than that of male rats.

The present findings also demonstrated that cocaine increased accumbal P-CREB levels in both male and female rats. However in male rats, P-CREB levels were elevated for 30 min after cocaine administration, while in female rats, P-CREB levels were elevated transiently for only 5 min. The increase in CREB phosphorylation of male rats is similar to findings by Walters and colleagues (2003) who demonstrated that P-CREB levels in the NAc were elevated 20 min after an acute cocaine administration. Behaviorally, while CREB may not be involved in the cocaine-induced motoric behaviors of male rats [179], it is unclear what role CREB phosphorylation plays in the cocaine-induced motoric behaviors of female rats. Based on the present findings, it is possible that the long persisting cocaine-induced ambulatory and rearing activities that are observed in female rats could be due to a more transient CREB phosphorylation in the

NAc. Sex differences in CREB phosphorylation could also affect cocaine addiction, as CREB phosphorylation has been shown to mediate cocaine reward and reinforcement. That is, enhancement in CREB phosphorylation can reduce cocaine conditioned place preference and self-administration [24;160]. Therefore, due to the role of CREB in cocaine reward processes, sex differences observed in CREB phosphorylation could underlie the disparities in cocaine-induced conditioned place preference and self-administation between male and female rats.

Cocaine produced a transient increase in P-CREB levels in the NAc of female rats. Although the mechanism underlying this effect is not known, it is possible that activation of PKA in the NAc of females could result in the activation of other feedback mechanisms (e.g., DARPP-32) that could modulate the phosphorylation of CREB. Alternatively, progesterone may have an effect on P-CREB levels in the NAc. Evidence suggests that progesterone can reduce P-CREB levels in hypothalamic nuclei and hippocampal cultures [63;122]. The rapid reduction in P-CREB levels in female rats maybe due the ability of progesterone to decrease CREB phosphorylation. Although this mechanism is not well understood, antagonism of progesterone receptor or prevention of progesterone to be metabolized can block the ability of progesterone to reduce P-CREB levels. This issue requires further investigation in the NAc of cocaine treated female rats to see whether the rapid P-CREB reduction in the present experiment is due to progesterone's presence in the brain.

In the present study, acute cocaine did not alter P-CREB levels in the CPu of male or female rats. The lack of cocaine effect on P-CREB levels was unexpected, as cocaine has been previously shown to increase P-CREB levels in the CPu of mice [79]. The

discrepancy in the findings could be due to two major methodological differences between the studies. First, the present study measured P-CREB levels using western blotting, whereas Kano and colleagues (1995) assessed P-CREB levels using immunocytochemistry. Alternatively, the different animal specie used in these studies may have contributed to the observed discrepancies. That is, the present study used Fischer rats while Kano et al (1995) used C3H/HeN mice. It is unclear whether these two animals have similar intracellular response to psychostimulants, which could make them a possible suspect for the differences found in the results.

The longer lasting ambulatory and rearing effects of acute cocaine in females could also be linked to differences in cellular response to the treatment. For instance, previous findings from our laboratory demonstrated that in the NAc of females, cocaine reduced levels of DA, DOPAC, HVA, and DOPAC/DA turnover, whereas in the NAc of males, cocaine only reduced DOPAC/DA turnover [51]. In the CPu, acute cocaine also reduced DOPAC/DA turnover of males, while having no effect in females. Furthermore, the sex disparities are demonstrated in mRNA expression, as accumbal D1 receptor mRNA levels were reduced in males 1hr after acute cocaine administration, but a similar change was not observed in females [50]. When considering the current and previous findings together, there appears to be dimorphism in the homeostatic process in response to acute cocaine administration between males and females. These differences could, in turn, assist in better understanding the mechanisms that render females more sensitive to cocaine than males.

Chapter 4: Sex differences in behavioral sensitization and tolerance to cocaine.

I. Introduction

Cocaine abuse in women is a growing problem in the United States. It is estimated that approximately 33% of all cocaine abusers are women [166]. Studies assessing women cocaine abusers have indicated that women begin cocaine use at an earlier age, become addicted faster, and enter treatment at an earlier age than men [62;98;115;181]. Women also have more emergency room visits following cocaine use [43]. Furthermore, cocaine related cues induce greater drug craving in women than in men [145]. Similarly, female rats have a greater locomotor response to acute and chornic cocaine administration [30;51;161;174;176], develop a more rapid acquisition to cocaine self-administration, reach a higher breaking point, and exhibit a greater response during the reinstatement phase than do male rats [103;104;148]. Female rats also develop a conditioned place preference to cocaine with fewer pairing session and lower doses of cocaine [153]. When considering the human and rodent literature together, it appears that females are more sensitive to cocaine's addictive properties than males.

Behavioral sensitization refers to the enhancement of a behavioral response after repeated exposure to psychostimulants that is greater than a previous acute exposure. In the past two decades, behavioral sensitization to cocaine in male rats has been intensely studied, as this model may play a crucial role in the neuroadaptations that is involved in processes that lead to cocaine addiction and craving [13;77;94;151]. Studies have shown that male rats exhibit behavioral sensitization to cocaine either after a single high dose or repeated injections [64]. In female rats, behavioral sensitization to cocaine has been

studied to a much lesser extent. Our group, as well as others, has shown that female rats exhibit a greater locomotor sensitization after repeated cocaine administrations [31;56;174]. However, the underlying mechanism for this enhanced response to cocaine sensitization is unclear.

Intracellular signal transduction mechanisms are responsible for the neuroadaptations that lead to behavioral sensitization [28;53;77;127;182]. A great deal of work has assessed the involvement of the cAMP system in the nucleus accumbens (NAc) and caudate putamen (CPu) on cocaine-induced behavioral sensitization. For example, chronic cocaine administration alters the cAMP dependent protein kinase (PKA) activity [34;168], which, in turn, enhances cocaine-induced behavioral sensitization [117]. Lastly, studies assessing the role of the transcription factor cAMP response element binding protein (CREB) in cocaine-induced behavioral sensitization have shown that $CREB^{\alpha\Delta}$ knockout mice exhibit an enhanced cocaine sensitization compared to their wild-type counterparts [179]. Based on the above-mentioned evidence, it is clear that various components of the cAMP pathway are influential in the neuroadaptations involved in cocaine-induced behavioral sensitization in male rats. Unlike the evidence available in male rats, no studies have assessed the role of the cAMP pathway in cocaine-induced behavioral sensitization. The aim of this study was to see whether the cAMP pathway could be involved in the sex differences in behavioral sensitization to cocaine. Findings from this inquiry could assist in better understanding the sex differences in cocaine addiction.

II. Method

Subjects:

60-day-old male and female Fischer rats (Charles River, Raleigh, NC) were individually housed in Plexiglas chambers (20 × 20 × 41 cm) layered with beta chips. Rats were given free access to standard lab chow and water and were maintained on a 12-hour light/dark cycle (lights on at 8:00 a.m.). All rats were weighed and handled for 5 consecutive days prior to experimental manipulations. Animal care and use was in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, Bethesda, MD) and approved by the Institutional Animal Care and Use Committee of Hunter College.

Drug and antibodies:

Cocaine hydrochloride was purchased from Sigma chemical Co. (St. Louis, MO). Cocaine solutions were prepared daily by dissolution in physiological saline (0.9%) and injected intra-peritoneally (i.p.) at a volume of 1 ml/kg. The primary antibody for PKA_C was purchased from Becton Dickinson Transduction Laboratories (Lexington, KY), antibody for P-CREB was purchased from Cell Signaling Technologies (Beverly, MA), and antibody for α-tubulin was purchased from Santa Cruz Technologies (Santa Cruz, CA). The anti-mouse and anti-rabbit secondary antibodies were purchased from Amersham Pharmacia (Piscataway, NJ).

Behavioral measurement:

All behavioral testing was performed in the home cage. Ambulatory and rearing activities were measured using a two frame automated Photobeam Activity System from San Diego Instruments (San Diego, CA). Ambulatory activity was determined by total counts of two consecutive photobeam interruptions in the lower frame. Rearing activity was represented as total counts of vertical motions detected by the upper frame.

To assess stereotypies, rats were videotaped for 40s using a handheld camcorder (30 cm away from the homecage) at 15, 30, and 45 min after the final saline or cocaine injection. The videotapes were later scored for stereotypies by three trained observers blind to each animal's treatment group. The rating for stereotypies was based on a modified version of the [35] scale (see Table 3). This scale consists of 10 ranked scores, ranging from 1 (given that the animal is asleep or inactive) to 10 (given that the animal exhibits splayed hind limbs). Throughout the study, a score of 10 was never observed.

Sensitization paradigm:

Male and female rats were randomly assigned into three treatment groups (n = 10/group): saline, acute cocaine, or chronic cocaine. Rats in the saline and acute cocaine groups were injected with saline (0.9%) twice daily for 13 consecutive days. Rats in the chronic cocaine group were injected with cocaine (15 mg/kg) twice daily for 13 consecutive days. In the morning of day 14, rats in the saline group were injected with saline, while rats in the acute and chronic cocaine groups were injected with cocaine (15 mg/kg). All injections were given in the home cage (first injection between 9 and 10 am,

and the second, between 5 and 6pm). Behavioral activities were measured for 60 min after the final drug treatment.

Protein measurements:

After decapitation (following a brief 20 s exposure to CO_2), rat brains were removed, flash frozen in 2-methylbutane (-40° C), and stored at -80° C until used. The CPu and NAc were dissected from coronal sections (1mm thick) and homogenized using a Polytron handheld homogenizer (Kinematica, Luzern, Switzerland) in a buffer containing HEPES 7.9 (20mM), KCl (10mM), EDTA (1mM), NP40 (0.2%), Glycerol (10%), NaCl (200mM), Pepstatin, Leupeptin, DTT (1M), Aprotenin, PMSF (100mM), NaF (50mM), & Na_3VO_4 (1mM). Total protein content was determined using a Bradford kit from Bio-Rad Laboratories (Hercules, CA). Protein samples (10 μg for PKA and 25 μg for P-CREB) were boiled in Lammeli buffer containing 1% β-mercaptoethanol, centrifuged, and loaded onto 10% SDS-PAGE. Gels were then electrophoresed, transferred to nitrocellulose membranes, and blocked for 30 min with 5% non-fat dry milk in tris-buffer-saline-tween (TBST) at room temperature. Membranes were probed overnight at 4°C with PKA_C (1:1000) or P-CREB (1:500) antibodies. After three washes with TBST, membranes were incubated with their appropriate secondary antibody (1:1000) for 60 min at room temperature followed by three more washes with TBST. Antibody binding was detected using an enhanced chemiluminescence kit (ECL; Amersham Pharmacia, Piscataway, NJ). Intensity of protein bands was quantified with a computer densitometer and Image Quant Program (Molecular Dynamics). For

normalization of protein levels, all membranes were re-probed with α-tubulin antibody (1:1000).

Statistical analysis of data:

Ambulations and rearing counts were analyzed using two-way repeated measures analysis of variance (ANOVAs) for the variables drug (saline, acute cocaine, & chronic cocaine), and time (12 five-min time blocks). In addition, one-way ANOVAs were used to determine differences within each time block. For Post hoc analysis, Tukey tests were conducted when appropriate. A Kruskal-Wallis ANOVA followed by Dunn's post-hoc tests were performed to assess the effects of acute and chronic cocaine on stereotypic behaviors. For analyzing protein levels, unpaired-samples t-tests were performed for pair-wise comparisons. Western blot data are presented as a ratio of PKA_C, or P-CREB over α-tubulin as arbitrary densitometric units. Determination of statistically significant differences was considered at the 0.05 probability level ($p < 0.05$).

III. Results

Sex differences in behavioral sensitization:

Overall, cocaine administration enhanced ambulatory counts in both male and female rats [Drug main effect, Males: $F(2,27) = 11.191$, $p < 0.001$; Females: $F(2,27) = 11.398$, $p < 0.001$). In male rats, chronic cocaine treated rats exhibited greater ambulations than the acute cocaine or saline treated rats [Drug × Time interaction, $F(22,286) = 2.260$, $p < 0.001$; Figure 10A]; where enhancements in activity were observed from 5 to 20 min (time blocks 2-4), 45 min (time block 9), and 50 to 60 min (time blocks

11-12) after cocaine administration (block 2: $F(2,27) = 9.277$, $p < 0.001$; block 3: $F = 20.014$, $p < 0.001$; block 4: $F = 10.362$, $p < 0.001$; block 9: $F = 12.925$, $p < 0.001$; block 11: $F = 17.232$, $p < 0.001$; block 12: $F = 13.070$, $p < 0.001$]. On the other hand, in female rats this effect was not observed (Figure 10B). In both male and female rats, rearing activity increased after acute and chronic cocaine administration [Drug main effect, Males: $F(2,27) = 9.052$, $p < 0.001$; Females: $F(2, 27) = 26.020$, $p < 0.001$]. While in male rats no significant differences were observed between acute and chronic cocaine treatment groups (Figure 11A), in female rats, rearing activity was reduced in the chronic cocaine group compared to the acute cocaine group [Drug × Time interaction, $F(22,297) = 3.155$, $p < 0.001$; Figure 11B]. That is, female rats treated with chronic cocaine exhibited lower rearing counts from 10 to 20 min (time blocks 3 & 4) compared to acute cocaine treated rats [block 3: $F(2,27) = 9.445$, $p < 0.001$; block 4: $F = 10.617$, $p < 0.001$]. When assessing stereotypic behaviors in male rats, acute or chronic cocaine did not enhance stereotypies of male rats (Figure 12). In female rats however, acute cocaine increased stereotypic behaviors compared to saline [$H = 7.2$, $p < 0.03$; Figure 10B]. No differences in the stereotypies between acute and chronic treated female rats were detected. Overall, neither acute nor chronic cocaine administration altered PKA_C or P-CREB levels in the NAc (Figure 13) or in the CPu (Figure 14).

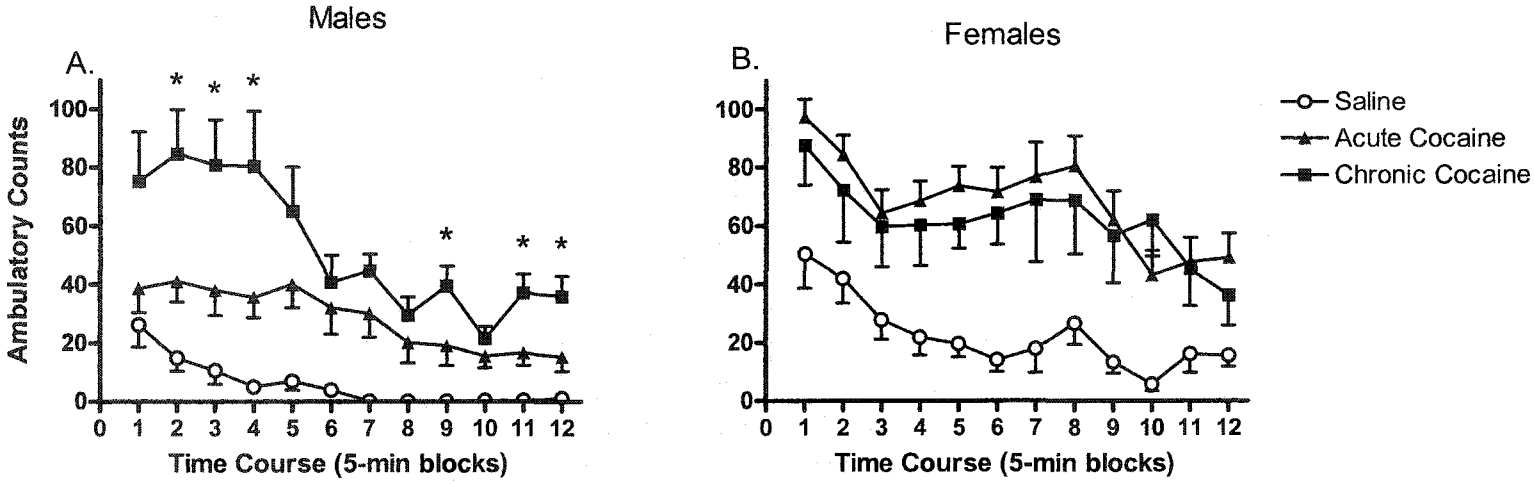

Figure 10: Time course of cocaine-induced ambulatory counts of male and female rats (n = 10/group). * Represents significant difference between chronic and acute cocaine treated male rats at a given time block.

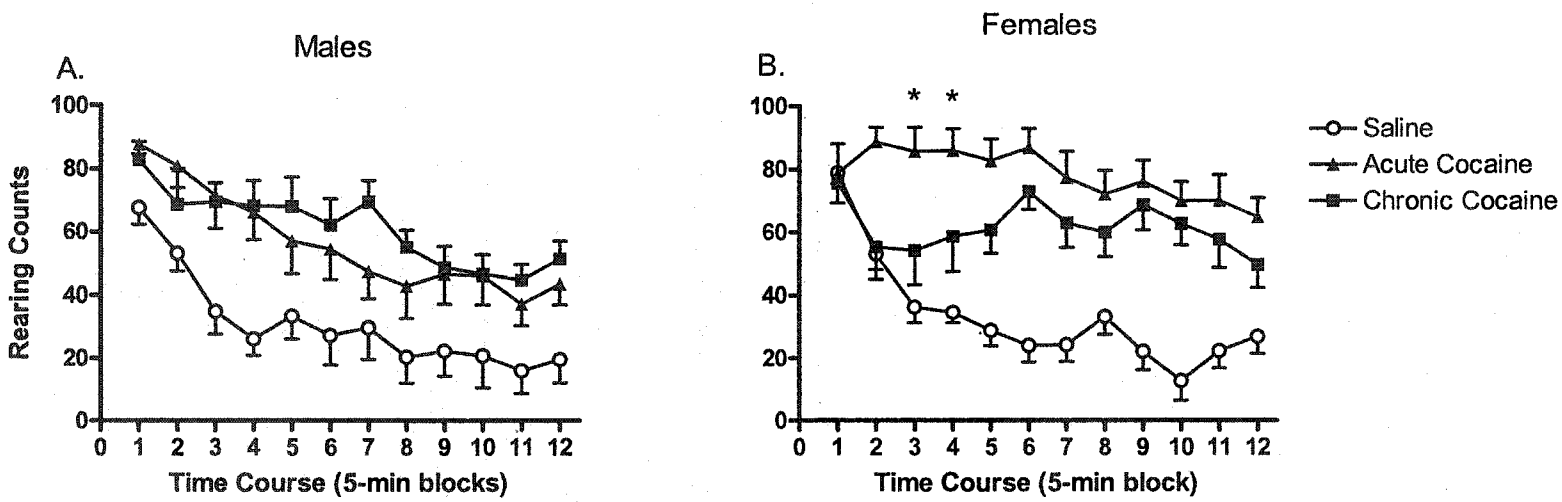

Figure 11: Time course of cocaine-induced rearing counts of male and female rats (n = 10/group). * Represents significant difference between chronic and acute cocaine treated female rats at a given time block.

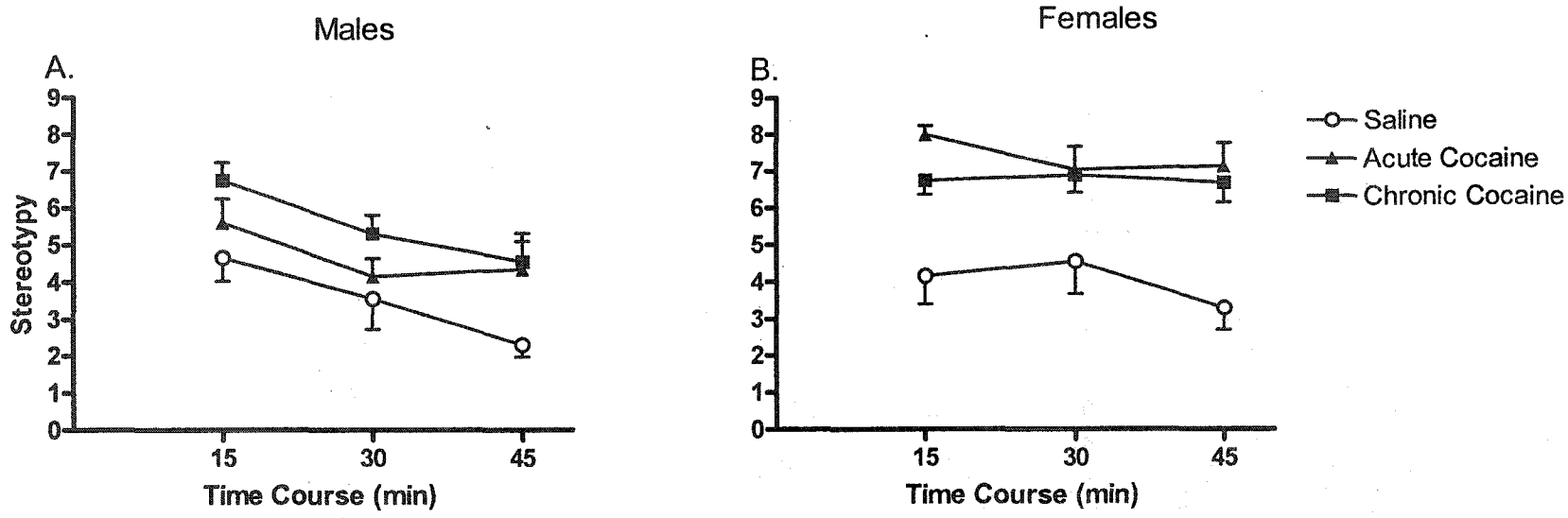

Figure 12: Median stereotypic scores of male and female rats measured 15, 30, and 45 min after cocaine administration (n = 10/group). Stereotypies were assessed for a duration of 40 s at a given time point.

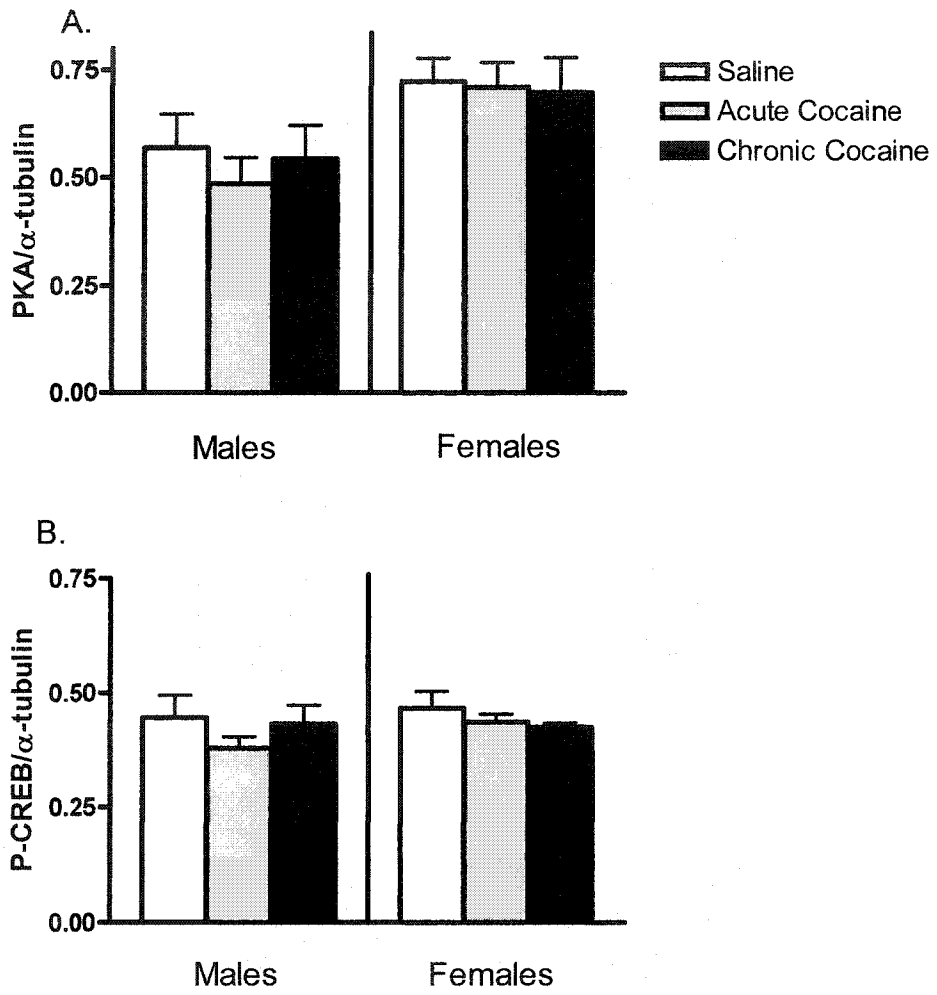

Figure 13: Mean (± S.E.M) densitomertic units of PKA_C (A), and P-CREB (B) levels in the NAc of cocaine treated male and female rats (n = 4/group).

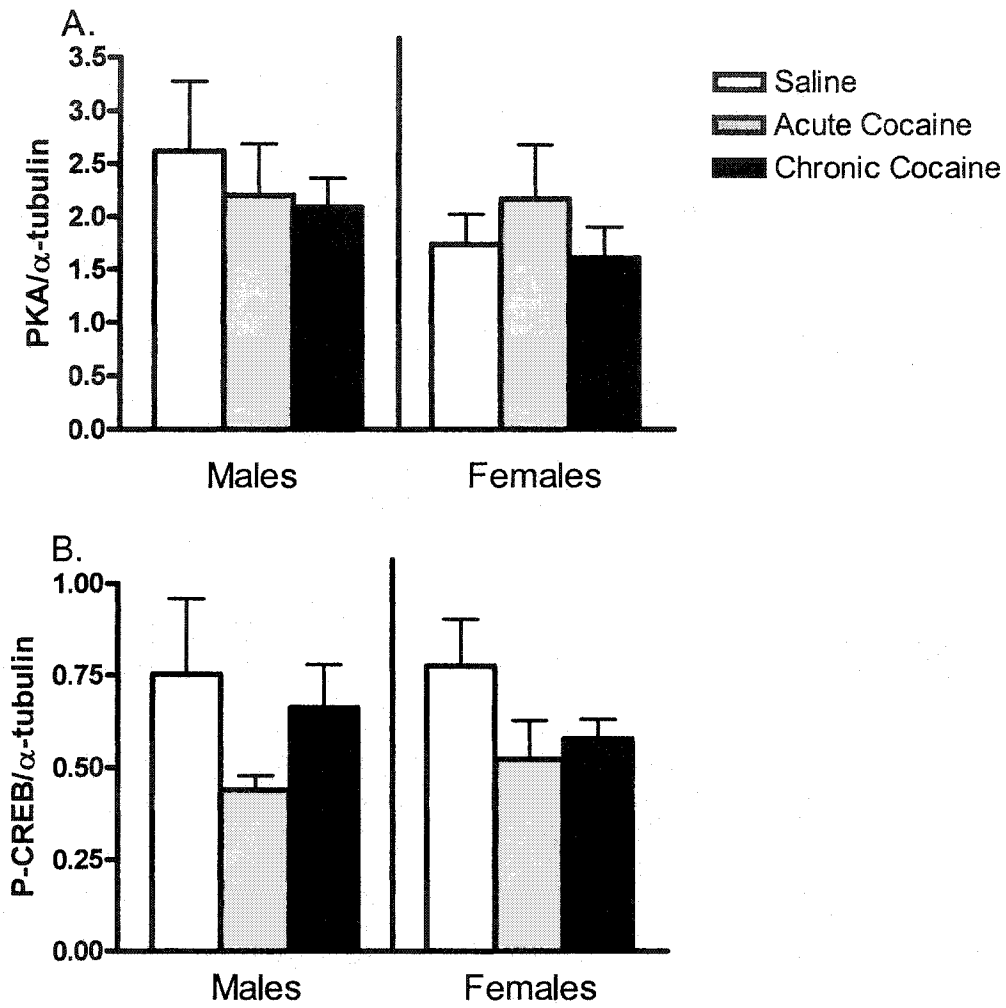

Figure 14: Mean (± S.E.M) densitomertic units of PKA_C (A), and P-CREB (B) levels in the CPu of cocaine treated male and female rats (n = 4/group).

Table 3. Modified stereotypy rating scale.

Score	Behavior
1	Asleep, inactive
2	Alert, actively grooming
3	Increased sniffing in one location
4	Intermittent rearing and sniffing
5	Increased locomotion and sniffing
6	Intense sniffing in one location
7	Continuous pivoting and sniffing
8	Continuous rearing and sniffing
9	Maintained rearing and sniffing for > 25s
10	Splayed hind limbs

IV. Discussion

The present study demonstrated sex differences after chronic cocaine administration. It was found that male rats exhibited sensitized ambulatory counts, whereas rearing counts were not sensitized. In female rats, the lack of sensitization to the ambulatory counts and tolerance to the rearing counts indicates that high doses of cocaine administered twice daily for 14 days results in behavioral tolerance to cocaine's psychomotor effects. No sensitized response to stereotypic activity was detected in male or female rats. Unlike the current findings, previous reports have shown that female rats sensitize to cocaine's psychomotor effects if the paradigm consists of fewer cocaine injections and/or lower cocaine doses compared to the present study [31;56;174]. Moreover, in male rats, a greater and more extended cocaine exposure is required to produce behavioral tolerance. For example, behavioral tolerance is achieved by continuous high-dose cocaine exposure for 7 or 14 days using osmotic minipumps [45;75;76;78;83;91;143]. Since female rats were able to develop behavioral tolerance with shorter cocaine exposure periods than male rats, it further attests that female rats are more sensitive to the psychomotor activating effects of cocaine than male rats.

The precise mechanism underlying the development of cocaine tolerance is unclear. However, it has been suggested that in male rats, development of cocaine tolerance is the result of an increase in the sensitivity of presynaptic DA autoreceptors in the CPu and a decrease in spontaneously active neurons in the substantia nigra [45;54;82;93;191]. Therefore, the appearance of cocaine tolerance in female rats could be due to sex differences in the functioning of the DA system. Indeed, female rats have a different DA tone in the CPu, as DA is released and uptaken faster than male rats [178].

It is not known what effect high doses of chronic cocaine administration has on the release and uptake of DA in female rats, but due to sex differences in the functioning of the DA system, disparities in DA tone could be a potential mechanism for the induction of cocaine tolerance in the present study.

There were no changes in PKA and P-CREB levels after acute or chronic cocaine administration in either male or female rats. Elevation in P-CREB levels have been previously linked to chronic cocaine administration paradigms [24;117]. However, studies that demonstrate changes in P-CREB levels use an administration paradigm that consisted of a withdrawal or abstinence period prior to the final cocaine injection. Therefore, it is likely that the reported P-CREB elevation is due to cocaine withdrawal-induced dysphoria. The administration paradigm of the present study did not possess an extended withdrawal/abstinence period, which may explain the lack of P-CREB elevation. Alternatively, the time-point of measurement may be responsible for the lack of P-CREB elevation. McClung and Nestler (2003) suggest that chronic cocaine administration produces an elevation in P-CREB levels within the first two weeks of injections, followed by the enhancement of a longer-lasting signaling protein, ΔFosB. A more careful time point assessment of cellular mechanisms underlying cocaine sensitization is needed. Taken together, our results suggest that sex differences in cocaine sensitization are not based on the PKA and P-CREB proteins at the time point that we assessed. Further investigation is required to elucidate the signaling mechanisms involved in the sex differences in chronic cocaine-induced behaviors.

Chapter 5: Conclusion

The focus of this dissertation was to investigate and understand the underlying mechanisms of the sex differences in response to acute and chronic cocaine administration. However, the findings of the present endeavors have produced more questions than answers. In summary, when measuring cocaine reward in female rats, the involvement of DA D1 receptors are more complicated that previously perceived, as the D1 antagonist SCH 23390 blocked cocaine CPP at lower doses but not at the high dose. On the other hand, all doses of SCH 23390 blocked the cocaine CPP of male rats. Results from acute cocaine-induced psychomotor effects were similar to previous findings from our group as well as others. Acute cocaine produced a longer lasting enhancement of ambulatory and rearing behaviors in female rats than male rats. Western blot analyses revealed that female rats have higher levels of basal PKA in the NAc than male rats. Moreover, although acute cocaine elevated P-CREB levels in the NAc of male and female rats, the effect was shorter lived in female rats than their male counterparts (5 vs. 30 min). Finally, chronic cocaine administration produced disparate psychomotor responses in male and female rats. Twice daily injections of cocaine for 14 days produce sensitization in the ambulatory counts of male rats, while the same injection paradigm produced tolerance in the ambulatory and rearing counts of female rats. In both male and female rats, PKA and P-CREB levels were unchanged in the NAc and CPu after chronic cocaine treatment compared to acute cocaine or saline treatments. When considering all of the findings as a whole, it becomes apparent that D1 receptors could at least partly be responsible for dimorphisms in cocaine reward, sex differences exist in the underlying signal transduction mechanism that are activated by cocaine, and that female rats are

more sensitive to cocaine than male rats after both acute and chronic cocaine administration.

Due to the complexity of the systems studied in this dissertation, it is possible that alternative mechanisms not assessed in the experiments could have produced the obtained results. For instance, at least one alternative factor could have caused the D1 antagonist to partially block cocaine CPP in female rats. That is, the doses of cocaine used to condition male and female rats differed. Male rats were conditioned with 20 mg/kg of cocaine, while female rats were conditioned with 5 mg/kg. These two doses produce approximately equal levels of CPP in male and female rats, although; it is unclear whether the two doses produce similar physiological effects in the rats. It is possible that the low dose of cocaine used in female rats may not activate or inhibit specific neuronal pathways or signal transduction mechanisms that the 20 mg/kg of cocaine could induce. Therefore, the D1 antagonist could produce different responses at a range of doses, similar to what was observed in the present study. This explanation is speculative and requires experimental verification.

In the present dissertation, acute cocaine produced a transient enhancement of P-CREB in the NAc of female rats that lasted for approximately 5 min. The disparity in the cocaine-induced P-CREB findings could be due to the intracellular signaling of the dopaminergic and the glutamatergic systems, both of which can regulate CREB phosphorylation independently and in interaction with one another. Stimulation of D1 receptors causes the phosphorylation of Thr-34 DARPP-32, which phosphorylates CREB, through inhibition of PP-1 [129]. Stimulation of D2 receptors causes the dephosphorylation of Thr-34 DARPP-32 by inhibiting cAMP formation and by

increasing intracellular Ca^{2+} [100;130]. Acute cocaine-induced accumbal P-CREB elevation is likely due to the phosphorylation of Thr-34 DARPP-32 via stimulation of D1 receptors (Figure 15). This mechanism is the candidate for describing the elevation of P-CREB in male rats, however; the mechanism behind the transient increase in P-CREB levels in female rats may differ. The precise mechanism for the transient P-CREB elevation is not clear in female rats, although speculations can be deducted based on the current evidence in the literature. First, it is possible that stimulation of D2 receptors could differentially inhibit P-CREB phosphorylation in female rats than in male rats. This could be made possible by D1 receptor desensitization or internalization. Specifically, behavioral studies indicate that female rats possess more sensitive D1 receptors [51], which could cause a faster desensitization or internalization of the receptor. Due to D1 receptor desensitization and its concomitant inability to stimulate cAMP, it is possible that D2 receptor inhibition of cAMP and enhancement of intracellular Ca^{2+} could cause a rapid reduction in P-CREB phosphorylation.

An alternative mechanism for transient P-CREB elevation in female rats could be due to the activation of PKA having a more robust effect in the dephosphorylation of Thr-34 DARPP-32 rather than phosphorylation of the protein. This could be made possible by the phosphorylation of NMDA receptors by the active PKA [26;52;164], thus increasing intracellular Ca^{2+}, which could result in the dephosphorylation of Thr-34 DARPP-32 by calcineurin. Dephosphorylation of Thr-34 DARPP-32 would then result in the disinhibition of PP-1, which would prevent or inhibit the phosphorylation of CREB. In addition, CREB phosphorylation can be promoted by a number of other signaling pathways. For example, the mitogen-activated protein kinase is also a possible

downstream effector of D1 and D2 receptors that could phosphorylate CREB [188;190]. More recent studies have also indicated the involvement of DA receptors in Akt signaling which could also phosphorylate CREB through a yet unidentified mechanism [9;20]. These intracellular signal transduction pathways are possible alternative mechanisms to for the acute cocaine-induced elevation of P-CREB in male and female rats. Moreover, these pathways could potentially function differently between male and female rats, which could render the sex differences observed in P-CREB levels.

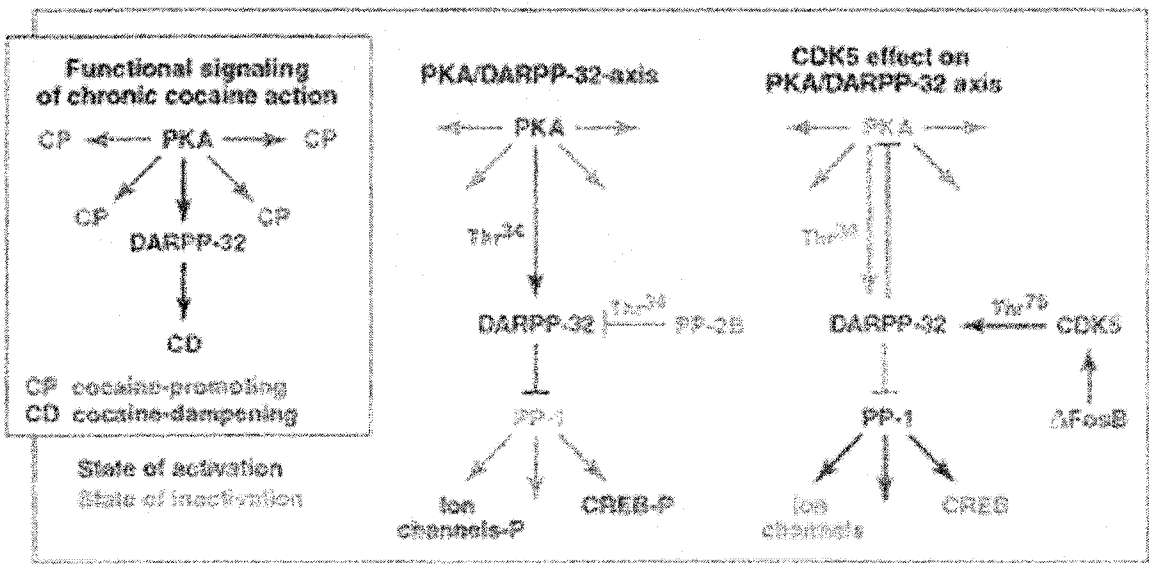

Figure 15: The involvement of DARPP-32 in CREB phosphorylation. DARPP-32 phosphorylation plays a bi-directional role in CREB phosphorylation. Phosphorylation of Thr-34 DARPP-32 results in the phosphorylation of CREB, while Phosphorylation of Thr-75 DARPP-32 results in the dephosphorylation of CREB.

The lack of P-CREB elevation after chronic cocaine administration is consistent with the model proposed by Nairn and colleagues (2004). Precisely, chronic cocaine upregulates the expression of cyclin dependent kinase 5, and this leads to increased

phosphorylation of Thr-75 DARPP-32 [14]. Phosphorylation of Thr-75 DARPP-32 causes the deactivation of PKA and the dephosphorylation of Thr-34 DARPP-32. As a result, a number of downstream proteins do not get phosphorylated, including CREB. The present dissertation did not find a change in P-CREB levels after chronic cocaine administration in male or female rats. Although speculative, this effect was likely due to the phosphorylation of Thr-75 DARPP-32, which could reduce or prevent phosphorylation of CREB. CREB phosphorylation has been shown to be a critical component for the development of behavioral sensitization. For instance, CREB knockout mice exhibit an enhanced behavioral sensitization and mice having an over-expression of CREB demonstrate a reduced or no behavioral sensitization compared to their controls [179]. Although the present finding did not find changes in P-CREB levels, it is necessary to point-out that it is in agreement with findings noted above. That is, as suggested by McClung and Nestler (2003), CREB phosphorylation is necessary for the development of sensitization, which occurs during the first several days of drug administration. During this period CREB phosphorylation is enhanced, thus leading to the transcription of a number of genes, including a gene from the Fos family known as ΔFosB. This protein is involved in maintaining and elevating the sensitization initially developed by cocaine and CREB. Thus, the lack of findings in the present study is likely due to P-CREB levels being measured after the critical period where CREB is elevated. An experiment demonstrating the elevation in ΔFosB in the samples tested would validate this suggestion.

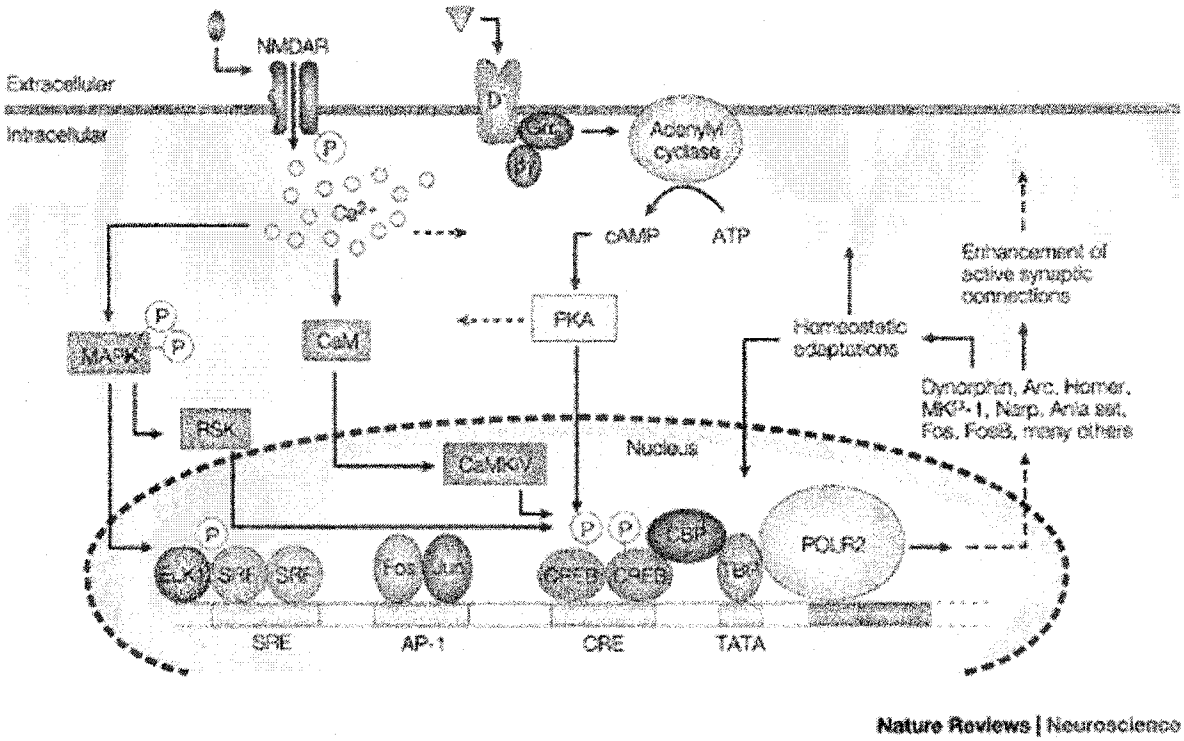

Figure 16: Intracellular transduction pathway for D1 dopamine and NMDA receptors. Stimulation of the D1 receptor causes the activation of several intracellular molecules that leads to gene transcription and cellular changes. (Adopted from Hyman & Malenka 2001).

The glutamate system has been implicated in the behavioral and intracellular actions of cocaine (Figure 16). To be precise, NMDA receptors have been studied for their role in behavioral sensitization and CREB phosphorylation. For example, antagonism of NMDA receptors prevents the development of behavioral sensitization to cocaine in male rats [67;81;99;186]. These receptors can also phosphorylated CREB via different mechanisms. More specifically, NMDA receptors can phosphorylate CREB both directly and indirectly. Direct CREB phosphorylation occurs by an increase in intracellular Ca^{2+}, which is a result of the phosphorylation of the NR1 subunit of the NMDA receptor by either PKA or depolarization of the cell [44]. Indirectly, NMDA

receptors phosphorylate CREB by the phosphorylation of Thr-34 DARPP-32, which is induced due to an increase in intracellular Ca^{2+}. Although, it is believed that NMDA receptors and their related signal transduction are necessary components for the development of behavioral sensitization. The present understanding of NMDA receptors in female rats and their role in cocaine-induced behaviors are not known. However, the ability of NMDA receptors to phosphorylated CREB could be one of the possibilities to why female rats respond greater to cocaine than male rats.

The present dissertation found that female rats had greater basal PKA levels in the NAc than male rats. This finding is of significant importance, as all the intracellular signaling pathways mentioned above are modulated by PKA. Therefore, having greater PKA could result in having different functioning of the cAMP, DARPP-32, and NMDA signaling. Higher PKA levels could also suggest the different functioning or sensitivity of DA receptors, as these receptors are coupled to G-proteins that activate PKA. Taken together, it is highly possible that the acute cocaine-induced transient P-CREB elevation in female rats could be the result of high PKA levels that produce a different signaling characteristic within the cAMP, DARPP-32, or NMDA systems. In addition, different signaling characteristics could also be involved in chronic cocaine induced tolerance that is observed in female rats. Unfortunately, very little is known about the intracellular signaling involved in cocaine tolerance, therefore, it is unclear which signaling pathways would be most influenced by the high PKA levels.

The studies conducted in this dissertation focused on gross sex differences without studying and/or measuring gonadal hormone effects. However, it is important to consider the gonadal hormones as a significant modulator of physiological effects in

female rats. Studies have shown that estrogen and progesterone in normal cycling and hormone replaced ovariectomized female rats can alter DA release, DAT densities, DA receptor levels, and signal transduction mechanisms. Due to the effects of estrogen and progesterone on various cellular mechanisms, cocaine-induced changes observed in female rats become particularly difficult to interpret and compare to male rats. Therefore, studying gross sex differences is a preliminary step in unraveling the underpinning of the disparities observed in acute and chronic cocaine effects.

The purpose of the research conducted here was to better understand the mechanisms by which female rats respond to cocaine. Ultimately, the findings of these studies are aimed at advancing the current knowledge in the way women become addicted to cocaine and what therapeutic approaches could best benefit female cocaine addicts. Due to the large amount of data demonstrating clear sex differences in the way the female brain responds to cocaine, it is critical for the scientific community to develop treatments that are specifically tailored for females.

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