Cocaine Shifts the Estrus Cycle Out of Phase and Caffeine Restores It

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Background: Sex differences in cocaine abuse are well established. Females have a higher sensitivity and thus higher vulnerability to cocaine abuse compared to males. There are many studies showing that sensitivity to cocaine reward varies during the estrus cycle.

Methods: Vaginal smears were examined through a DIFF staining kit and viewed through a microscope to determine the estrus cycle stage. Smears were taken immediately before and after cocaine and/or caffeine injections. Furthermore, we suggest a new tool to analyze the estrus cycle by using electrical resistance of the vaginal mucosa.

Results: In the present study, we discovered that cocaine directly induced changes in the estrus cycle. Interestingly, caffeine did not affect the estrus cycle and nor did the combination of cocaine and caffeine. We observed that caffeine blocked the cocaine-induced estrus cycle changes using conventional exfoliate cytology. Therefore, caffeine may have neuroprotective properties on the changes induced by cocaine.

Conclusion: These phase changes in the estrus cycle may be the underlying cause of sex differences in cocaine addiction that can be blocked by caffeine. Thus, we propose a valuable insight into sex differences in cocaine abuse and reveal a possible treatment with antagonizing the adenosine system.

Introduction

COCAINE ABUSE IS SEEN in more than 1.8 million Americans, and the number of women users is increasing at an alarming rate.¹ Given the highly addictive properties of cocaine, this phenomenon has become a public health concern. Cocaine significantly increases neurotransmitters, such as dopamine (DA) and serotonin, in the reward areas of the brain.² More and more evidence is pointing to the increased vulnerability of cocaine abuse in women compared to men.³ In laboratory animals, females self-administer cocaine more rapidly, become dependent earlier, and relapse faster compared to males. However, the reason for these sex differences remains uncertain.

Sex hormones, such as estrogen, are regarded as the underlying cause. Estrogen levels fluctuate throughout the four stages of the estrus cycle.⁴ Laboratory animals with higher estrogen levels also have higher sensitivity

to cocaine. Thus, during the estrus cycle stages when estrogen levels peak, females are more susceptible to cocaine. For instance, females show a higher stimulatory response to lower doses of cocaine compared to males.^{3,5} The level of sex hormones fluctuates throughout the four stages of the estrus cycle, which alters sensitivity to cocaine.⁴ Human studies showed higher cocaine stimulatory response during the luteal phase (moderate estrogen levels, high progesterone) and not the follicular phase (low estrogen levels and progesterone).^{6,7} Throughout the estrus cycle in rats, the highest estrogen levels are during the proestrus phase, and these levels then drop during the estrus and diestrus phase.⁸ Additionally, ovariectomy (OVX) procedures significantly diminish these sex differences.

Adenosine is one system that is associated with the sleep–wake cycle and is most important in regulating neuronal activity.⁹ Thus, more and more evidence is pointing to its involvement in regulating DA release.¹⁰

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Caffeine is a psychostimulant that promotes wakefulness by nonselectively antagonizing the adenosine A1 receptors (A1R) and A2 receptors (A2AR). There are observed interactions between the adenosine system and DA.¹¹ Therefore, the adenosine system may regulate cocaine reinforcement.

Previous studies in our lab using the BRODERICK PROBE[®] biosensor showed that caffeine combined with cocaine reduced the significant increase of DA that is associated with cocaine.^{12,13} The biosensors were surgically implanted in the nucleus accumbens, which involves the reward areas of the brain. DA is a key factor in cocaine-seeking behavior, causing the euphoric feeling of reward. This effect was more sensitive for females and occurred at lower doses compared to males, supporting the increased vulnerability associated with females.¹⁴ Caffeine administration with cocaine decreased the cocaine-induced increase in DA. Since caffeine acts on adenosine receptors in the brain, this suggests a role for drug therapies that target adenosine receptors for treating cocaine addiction.

These findings show that caffeine may protect the limbic neurons from the addictive properties of cocaine. The present study set out to examine the sex differences caused by cocaine and the neuroprotective behavior of caffeine using the estrus cycle.

Methods

Subjects

Adult female Sprague–Dawley rats (*rattus norvegicus*), 2 months of age, were purchased from Charles River Laboratories. Animals were housed in the Animal Care Facility under the auspices of the City College Institutional Animal Care and Use Committee (IACUC) in compliance with National Institutes of Health (NIH) guidelines. Food (Purina Rat Chow) and water were available *ad libitum*. Females were housed separately from males in airtight seclusion, two or three per chamber.

Drugs and doses

Studies were performed in three groups: cocaine, caffeine, or a combination of cocaine and caffeine, with low, middle, and high doses. Each group had a sample size of eight. Within the three groups, each animal received each dose as a within-subject control. The different doses were given at least 48 h after the previous one to eliminate residual drug effects. Doses were given as described in Table 1 in pseudo-random order. Solutions for cocaine HCl (Sigma-Aldrich) and caffeine (Sigma-Aldrich) were dissolved in distilled water and injected intraperitoneally (i.p.). Saline (0.9% NaCl) solution was used as a control. Doses were chosen based on peak effects from the literature^{15–17} and previous NMI experiments done in this laboratory to correlate behavior with biochemistry.^{12,13,18}

TABLE 1. DOSES ADMINISTERED I.P. IMMEDIATELY BEFORE THE INJECTION TRIAL

Group	Dose
Cocaine (<i>n</i> = 8)	Saline LD: 2.5 mg/kg MD: 5 mg/kg HD: 10 mg/kg
Caffeine (<i>n</i> = 8)	Saline LD: 12.5 mg/kg MD: 25 mg/kg HD: 50 mg/kg
Combination (<i>n</i> = 8)	Saline LD: 2.5 mg/kg coc and 12.5 mg/kg caff MD: 5 mg/kg coc and 25 mg/kg caff HD: 10 mg/kg coc and 50 mg/kg caff

i.p., intraperitoneally; coc, cocaine; caff, caffeine; LD, low dose; MD, middle dose; HD, high dose.

Vaginal smears using exfoliate cytology

Samples were collected before and after injections within a 30 min period. Females were swabbed with a saline-soaked cotton tip and dotted on a microscope slide. Samples were observed at 40× objective lenses using an Invert Research Microscope (Nikon Eclipse TI) Zeiss Axioplan 2 Imaging and CariZeiss Vision GmbH. The vaginal smears were stained with a Diff staining kit (IMEB, Inc.) and analyzed according to four stages: proestrus, estrus, metestrus, and diestrus.¹⁹ Samples were assessed based on the proportions of the type of cells to determine the appropriate stages. There were three types of cells: round nucleated epithelial cells, irregular shaped anucleated cornified cells, and little round leukocyte cells. The proestrus phase had prominently epithelial cells. The estrus phase had cornified cells. The metestrus phase had an equal amount of all three cells. Finally, the diestrus phase consisted of predominantly leukocytes.

Electrical resistance

Estrus cycle stages were studied by measuring the electrical resistance of the vaginal mucosa (EC40; Fine Science Tools). Subjects were held in hand on their backs, and a probe was placed in the vaginal opening as a recorder calculated the electrical resistance. This was performed before and after injection with 2.5 mg/kg cocaine and saline and prior to the vaginal smear swab. The cocaine low dose data are shown in Table 2, and the saline data are given in Table 3. Statistical analysis was performed on the difference between pre- and postelectrical resistance for the two treatments. A one-way analysis of variance (ANOVA) and *post hoc* Bonferroni test were performed. Alpha significance was set at $p < 0.005$. Preliminary data have been collected on

TABLE 2. VAGINAL SMEARS AND ELECTRICAL READINGS TAKEN FOLLOWING A LOW DOSE (25 MG/KG I.P.) OF COCAINE BEFORE AND AFTER INJECTIONS

Physiology		Electrical reading	
Pre	Post	Pre	Post
Metestrus	Diestrus	1.8	0.8
Diestrus	Proestrus	2.2	1.4
Early diestrus	Metestrus	2.6	0.8
Metestrus	Diestrus	2.6	1.4
Proestrus	Estrus	2.1	3.7
Metestrus	Estrus	1.4	2.1
Metestrus	Estrus	2.2	4.8
Metestrus	Diestrus	0.7	0.8
Metestrus	Diestrus	1.7	1.1
Metestrus	Proestrus	2.3	0.6
Proestrus	Estrus	2.4	1.5
Estrus	Diestrus	3.2	1.8
Late diestrus	Late proestrus	1.5	2.3

caffeine and combination groups using electrical resistance, showing a trend consistent with vaginal smears, but more data are needed.

Results

Vaginal smears

Vaginal smears were assessed using a microscope and scored based on cells described in the Methods section (Figs. 1A–H and 2A–H). For all doses of cocaine, female smears showed differences in the estrus cycle phase before and after injections within 30 min. Cocaine induced seemingly random changes in the estrus cycle (Figs. 1C and D and 2C and D). This was not seen following caffeine administration (Figs. 1E and F and 2E and F). Furthermore, combination of cocaine and caffeine did not alter the estrus cycle (Figs. 1G and H and 2G and H). The cocaine-induced changes were blocked by caffeine.

Electrical resistance

Cocaine injections showed variability in the electrical resistance of the vaginal mucosa that was not seen following

TABLE 3. VAGINAL SMEARS AND ELECTRICAL READINGS TAKEN FOLLOWING SALINE (BODY WEIGHT/KG I.P.)

Physiology		Electrical reading	
Pre	Post	Pre	Post
Diestrus	Diestrus	0.8	0.6
Diestrus	Diestrus	0.7	0.6
Late estrus	Late estrus	2.5	2.3
Early diestrus	Late diestrus	1.3	1.3
Early metestrus	Late metestrus	1.6	1.6
Estrus	Estrus	2.9	3.0
Estrus	Estrus	3.1	2.9
Estrus	Estrus	2.5	1.8

FIG. 1. (A–H) Studies on the estrus cycle in females show that cocaine induces changes in the estrus cycle that is not seen with caffeine, a combination of cocaine and caffeine, or saline. These changes are even expressed at the low dose as seen above.

saline injections (Tables 2 and 3). A one-way ANOVA showed a treatment significance between the cocaine and saline group, $F(1, 20) = 16.498$, $p < 0.000619$. A Bonferroni test revealed cocaine significantly changed the electrical resistance before and after injections that was not seen following saline ($p = 0.000619$). These results were consistent with the cocaine-induced estrus cycle stage changes.

Discussion

There is an abundance of research on the influence of sex hormones on cocaine addiction. Laboratory animals with higher estrogen levels were shown to have higher sensitivity to cocaine.^{5,20,21} Estradiol studies have shown an increase in cocaine effects in the locomotor and motivational aspect in cocaine addiction.^{4,22} Thus, the most assumed theory is that estrogen is potentially essential in the reinforcement of cocaine. However, there are still conflicting results

FIG. 2. (A–H) Studies on another batch of animals showed that cocaine induced changes in the estrus cycle that was not present in the other groups.

in the literature. Some report cocaine responses are increased during the estrus phase,^{23,24} had the most self-administration during the proestrus phase,²⁵ or exhibited no differences between the estrus or proestrus phases.^{26,27}

Our findings showed that cocaine directly changed the estrus cycle, and thus changed estrogen levels for females, affecting their sensitivity. Contrary to the effects of cocaine, caffeine did not cause such changes and blocked the effects of cocaine when taken together. This pattern of neuroprotection via caffeine was similar to responses in previous studies of DA release,¹³ conditioned place preference,²⁸ locomotor activity,^{18,29} and acoustic startle response,¹⁴ suggesting that DA, adenosine, and estrogen strongly interact with each other, causing increased sensitivity to cocaine that was reversed when the adenosine system was antagonized.

There is another method of analyzing the estrus cycle phase changes. This method interestingly reads the electrical resistance of the vaginal mucosa. Results showed a correlation between the estrus cycle phase and voltage.²⁸ Cocaine changed the electrical resistance (kilohm) at low dose, whereas saline did not significantly change the electrical resistance. Interestingly, preliminary data show that caffeine and a combination of cocaine and caffeine do not change the electrical resistance as well, but more data are needed. The electrical resistance voltage and vaginal smear exfoliate cytology show parallel results. However, the volt reader gives a conclusive number immediately after, whereas a vaginal smear is subjectively scored. Therefore, this report on the electrical resistance of the vaginal mucosa is a new tool to test/evaluate estrus cycle changes better.

In addition to hormone effects, there may be other factors involved in these sex differences.⁵ Differences in signaling caused by sexually dimorphic regulation of DA or other neuronal regulators may be the origin of these differences. Also, differences caused by the sex chromosomes on the nigrostriatal or mesocorticolimbic system could play a role.

Conclusion

These results have important potential clinical relevance in the treatment of cocaine addiction for females. Cocaine addiction is a multifocal issue, encompassing biological, psychological, economic, and social concerns. Our work strives to contribute to an understanding of the basic pharmacological mechanisms of cocaine actions so as ultimately to develop treatments for cocaine addiction. As there is no one definitive treatment, a better understanding of the mechanisms contributing to cocaine abuse will be a key step in finding an answer. We provide evidence on the direct interaction between cocaine and the estrus cycle. Furthermore, we provide potential insight/options for the adenosine system playing a key role in treating cocaine addiction.

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Author Disclosure Statement

No competing financial interests exist.

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