

## Nicotine Incites a Transformation to Estrus from any Estrous Cycle Stage

Leslie Wenning<sup>1,4\*</sup>, PA Broderick<sup>1,2,3,4</sup> and R Philip<sup>1,2,5,6</sup>

<sup>1</sup>Department of Molecular, Cellular and Biomedical Sciences, CUNY School of Medicine, The City College of New York, New York, NY USA

<sup>2</sup>Department of Biology, CUNY Grad. Ctr., New York, NY USA

<sup>3</sup>Department of Neurology, NYU Langone Medical Center and Comprehensive Epilepsy Center, New York, NY USA

<sup>4</sup>Department of Anesthesiology, NYU Langone Medical Center, New York, NY USA

<sup>5</sup>Dartmouth College, Hanover, NH USA

<sup>6</sup>The Bronx High School of Science, Bronx, NY USA

\***Corresponding Author:** Leslie Wenning, c/o Lab of Dr. Patricia Broderick, Department of Molecular, Cellular and Biomedical Sciences, CUNY School of Medicine, The City College of New York, 160 Convent Avenue, New York, NY, USA.

R Philip was a high school student at The Bronx High School of Science when she conducted research for this paper.

**Received:** May 16, 2017; **Published:** June 03, 2017

### Abstract

Females are significantly more likely to become addicted to nicotine as demonstrated by studies reporting that women have greater difficulty quitting smoking compared to men. Intensive research focuses on nicotinic acetylcholine receptors as the agents responsible for nicotine addiction. However, the mechanism by which females are more susceptible to nicotine dependency is not fully understood. In order to address these sex differences in response to nicotine, estrogen and progesterone levels of 6 female Sprague Dawley rodents, *Rattus norvegicus*, were monitored at the following time intervals: pre-nicotine injection, 30 minutes post-6 days of daily nicotine injection, and one hour post-6 days of daily nicotine injection. Exfoliate cytology of vaginal mucosa was concurrently sampled at these three time periods, stained, and analyzed via microscopy. The number of nucleated epithelial cells, anucleated cornified cells, and leukocytes was estimated to determine estrous cycle phase: proestrus, estrus, metestrus, or diestrus. Animals injected with saline were used as a control. Injections were administered intraperitoneally and separated by at least 24 hours to eliminate residual drug effects. Before nicotine injection, animals were in various estrous cycle stages with no significant difference in the number of animals per stage. Thirty minutes post-6 days of nicotine administration, a significantly greater number of non-prescient mammals transitioned into estrus from their original stage rather than transitioning into the subsequent cycle stage ( $p < 0.001$ ). One hour post-6 days of nicotine administration, these values remained significant as more animals transitioned into estrus at this time interval ( $p < 0.001$ ). Correspondingly, an overwhelming majority of nucleated epithelial cells became anucleated cornified cells at both 30 minutes and one hour post-6 days of daily nicotine injection. Animals injected with saline remained in the same stage throughout administration. Our results demonstrate that nicotine incites a transformation to estrus in the murine model, regardless of cycle phase before nicotine administration. During the estrus stage, both estrogen and progesterone were low as confirmed by an increase in nucleated cells on vaginal smear. Due to the fact that nicotine administration was associated with a decrease in ovarian hormones, a lack of estrogen and progesterone could be paramount to elucidating the sexual dimorphism of addiction.

**Keywords:** Addiction; Estrogen; Estrous Cycle; Estrus; Nicotine; Progesterone

### Abbreviations

Acetylcholine Receptors (AChRs); Dopamine (DA); Follicle Stimulating Hormone (FSH); Intraperitoneal (IP); Knockout (KO); Luteinizing Hormone (LH); Knockout (KO) of Estrogen Receptor  $\beta$  (ER $\beta$ ) are ( $\beta$ ERKO); Nicotinic Acetylcholine Receptors (NACHRs); Serotonin (5-HT)

**Introduction**

**The Estrous Cycle**

Females are significantly more likely to become addicted to nicotine as demonstrated by studies reporting that women have greater difficulty quitting smoking compared to men [1,2]. Thus, in order to delve into sex differences in addiction, the non-priscent mammalian estrous cycle was examined. The murine estrous cycle typically lasts 4 to 5 days and is comprised of 4 stages: proestrus, estrus, metestrus, and diestrus. During proestrus, estrogen and estradiol reach peak amounts as progesterone declines during ovarian follicle growth. On vaginal smear, nucleated epithelial cells including parabasal cells may be visualized. During estrus, LH increases as estrogen decreases. Progesterone remains low. These hormonal changes produce mature anucleated cornified cells on vaginal smear. During metestrus, estrogen remains low, while LH and FSH increase. On vaginal smear, anucleated cornified cells, leukocytes, and nucleated epithelial cells are predominant. The subsequent diestrus stage may be divided into two stages as there is an increase in the number of nucleated cells towards the end of this stage. During diestrus, progesterone is high and FSH is low, which results in leukocytes on vaginal smear. As diestrus transitions into proestrus, FSH increases as ovarian follicles grow.

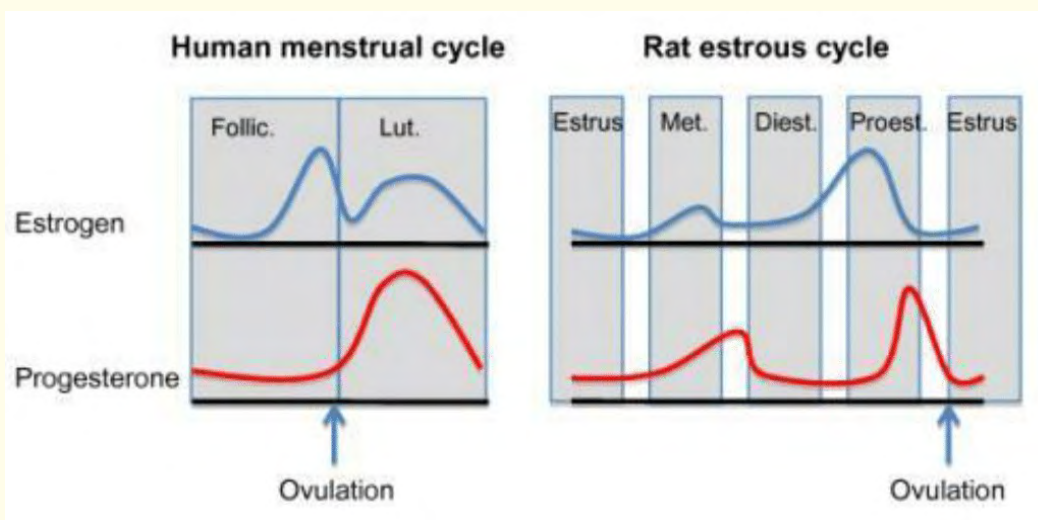


Figure 1: The human menstrual cycle versus non-priscent mammalian estrous cycle [3].

**Sex Differences in Nicotine Dependency**

Nicotine is a powerful parasympathomimetic and nicotinic acetylcholine receptor agonist that opens ligand-gated ion channels for rapid synaptic neurotransmission (Figure 2).

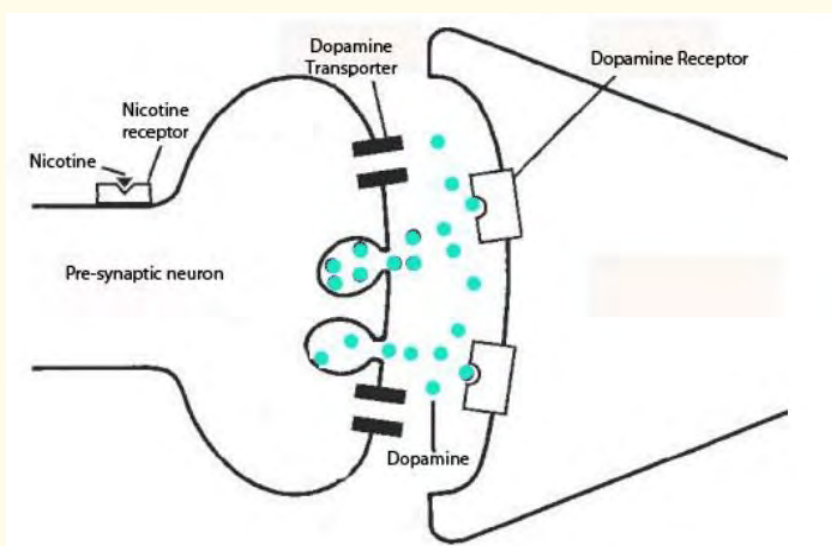
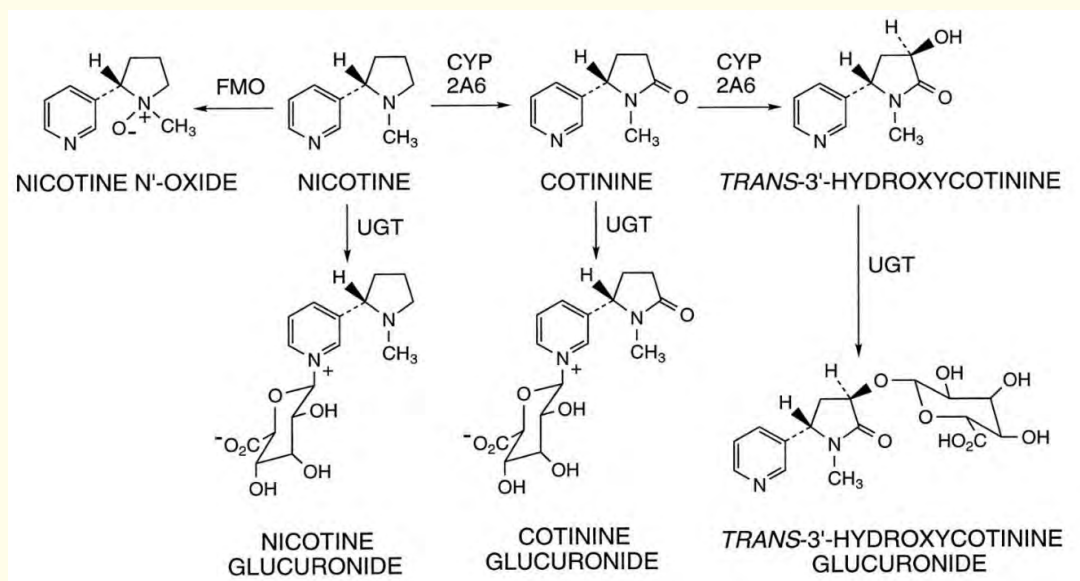


Figure 2: Nicotine molecules mimic acetylcholine, binding to NACHR, which causes the associated ion channel to open in the parasympathetic nervous system.

Nicotine is mainly metabolized to the active metabolite cotinine that is further processed to trans-3'-hydroxycotinine [4].



**Figure 3:** The nicotine pathway displaying the metabolism of nicotine to active metabolites, cotinine and trans-3'-hydroxycotinine [5].

NACHRs control the periphery's muscular reaction to a stimulus. They transmit the nerve impulses to motor neurons, affecting an action, and transmit across synapses in the autonomic ganglia [6]. Nicotinic receptors are present on the neurons of the ventral tegmental area, which terminate in the nucleus accumbens. Both the nucleus accumbens and ventral tegmental area are central to the reward circuit, so their relationship with nicotinic receptors is fundamental to understanding nicotine addiction. Nicotine may also bind to nicotine receptors on the aforementioned neurons, exciting the cell and increasing the concentration of DA [7].

NACHRs are intensely studied in the context of nicotine addiction. By mimicking the parasympathetic nervous system, nicotine increases the amount of DA in circulation. This increase in DA occurs via a cholinergic-DAergic link, as nicotine acts on AChRs and increases cholinergic receptor activity [7]. Because of the binding of nicotine to nicotine receptors (Figure 2), the amount of DA released from the presynaptic neuron increases, resulting in the sensation of reward that is associated with nicotine addiction and may even enhance the addictive effects of other alkaloids such as cocaine when used in conjunction with these substances of abuse [8]. When DA increases, the substantia nigra decreases DA production and both the raphe nuclei and enterochromaffin cells decrease 5-HT production. Once the cell has become excited and the channel closes, the nicotinic receptors become temporarily unresponsive to any neurotransmitters. Continual exposure to nicotine is artificially prolonged, leading to the desensitization that causes smokers to develop a tolerance for nicotine. Moreover, some researchers propose that estrogen promotes a greater sensation of reward during nicotine use in females by augmenting nucleus accumbens DA release. Furthermore, they hypothesize that women experience more difficulty during smoking cessation compared to men because Corticotropin-Releasing Factor stress systems are sensitized to promote the inhibition of DA release during withdrawal in females [2]. Finally, in a study examining the effect of gender and cigarette smoking on reactivity to psychological and pharmacological stress provocation, the results suggested that women may be more sensitive than men to nicotine's impact on cortisol response [9].

Although smoking is declining in the American population, this decrease is occurring less rapidly in women as demonstrated by the fact that lung cancer caused by smoking tobacco became the leading cause of cancer, overtaking breast cancer in women [10,11]. As a result, female smokers may outnumber their male counterparts in the next decade [11]. Unfortunately, sex differences related to nicotine dependency are complex. Research demonstrates that females may experience more conditioned positive reinforcement by non-nicotine stimuli such as the social interaction provided by smoking with others and the sensory stimulation of seeing and smelling smoke [11]. Correspondingly, in a review of the literature by the National Institute on Drug Abuse, women have greater difficulty quitting smoking than men, especially when using nicotine replacement therapies such as nicotine gum or patches [11,12]. Indeed, the fact that nicotine replacement therapies are less successful in women suggests that non-nicotine factors add to the difficulty of females abstaining from nicotine. However, despite evidence suggesting that conditioned reinforcement to non-nicotine variables may underlie female vulnerability to nicotine addiction, there are studies demonstrating greater female dependence on the chemical properties of nicotine itself.

As previously mentioned, estrogen may promote greater DA release in response to nicotine use [2]; and at certain doses, female non-priscent mammals self-administer more nicotine than males [13]. Similarly, human research suggests that females are more sensitive to the reduction in negative affect and body weight that is associated with smoking [1]. The feminine predisposition to nicotine addiction is further supported by research suggesting that estrogen may promote vulnerability to nicotine addiction, while progesterone may actually protect against nicotine dependency [14]. Moreover, estrous cycle phase may affect withdrawal symptoms during cessation. Thus, natural hormonal fluctuations that occur during puberty, pregnancy, and menopause, as well as artificial hormonal fluctuations caused by medications such as birth control pills, should be taken into consideration when interpreting female susceptibility to nicotine.

In the Broderick Lab's body of work on alkaloids, the parasympathomimetic effects of cocaine and caffeine on neurotransmitter activity were studied *in vivo* using the BRODERICK PROBE® [15-33]. As a natural expansion of this extensive work on the alkaloid family, the Broderick lab studied the impact of nicotine on the murine reproductive cycle in order to test the hypothesis that nicotine administration would affect estrogen and progesterone concentrations, thereby causing a change in the estrous cycle. Indeed, observing which stage of the estrous cycle is predominant after nicotine administration may reveal the relationship between nicotine and both estrogen and progesterone. To determine which estrous cycle phase predominates, the prevalence of anucleated cornified cells, nucleated epithelial cells, and leukocytes on vaginal smear may show which hormones are manipulated as nicotine is administered at varying dosages. In this manner, understanding the interaction between nicotine, estrogen, and progesterone may bring us closer to optimizing smoking cessation therapies for women.

### Prepulse Inhibition

Prepulse inhibition (PPI) is a suppression of the startle reflex when a weak stimulus, or a prepulse, precedes the startling stimulus [34]. For example, a subject, in this case a rodent animal, would receive the prepulse as a kind of preparation for the larger stimulus ahead, and would not be as startled as it would be when responding to a pulse without a prepulse. This inhibitory response is important for normal brain function as it prevents sensory overload. It should be noted that it is a natural physiological response for the nervous system to reduce the startle response to prevent cognitive overload. Thus, PPI further dampens the startle response and builds upon the nervous system's automatic suppression of the startle reflex. A further reduction from the normal neuronal sensory reduction in response to channel sensory information in the process of sensory gating may or may not be optimal. This further reduction may indicate that sensorimotor gating is dysfunctional. It may mean that the neuronal auditory network may result in cognitive dysfunction. Cocaine per se and schizophrenia related disorders including cocaine-induced schizophrenia result in a significant PPI reduction, particularly in females compared to males. This inability to reduce the impact of sensory flow may indicate that the amplitude of the startle response from the animal or human subject does not decrease but instead may increase [35].

The startle reflex operates via the auditory pathway through a network of sensorimotor gating processes in the auditory cortex, cochlear nuclei, and components of the mesocorticolimbic brain reward circuitry. Areas of the brain stem are crucial to the execution of the

startle response [26]. The acoustic startle paradigm tests whether PPI has been disrupted or not, and, therefore, is a model for anxiety and fear related disorders.

Based on previous research by the Broderick lab demonstrating the relationship between ovarian hormones and PPI during cocaine usage [26], other sex differences regarding drug use and abuse may also be related to these hormones. Indeed, fundamental sex differences were observed regarding PPI and startle reflex, as well as mesocorticolimbic and nigrostriatal neuronal circuitry. In agreement with the Broderick lab, previously cited, males generally have higher PPI than females [36]; and in agreement with the Broderick lab, previously cited, many studies conclude that ovarian hormones play a significant role in the response to auditory stimuli [37,38]. Finally, research has demonstrated a relationship between nicotine, PPI, and startle response [34].

## Methods

### Subjects

Adult female Sprague Dawley animals (210 - 230 kg; Charles River Laboratories, North Carolina) were housed in the Animal Care Facility under the auspices of the City College Institutional Animal Care and Use Committee in compliance with National Institute of Health guidelines. Food (Purina Rat Chow) and water were available *ad libitum*.

Nicotine (0.6 mg/kg, 0.8 mg/kg, 1.0 mg/kg, and 1.2 mg/kg) was introduced IP into female Sprague Dawley animals (260 – 300 kg). Nicotine hemisulfate salt (N1019 Sigma Aldrich), a mixture of the nicotine base and the nicotine sulfate, was dissolved in distilled water and administered daily for 6 days. Each animal received 0.6 mg/kg nicotine for 6 days, 0.8 mg/kg nicotine for 6 days, 1.0 mg/kg nicotine for 6 days, and 1.2 mg/kg for 6 days; and after each of the 4 nicotine dosing schedules, a vaginal smear was taken both 30 minutes and 1 hour after day 6. Injections were separated by at least 24 hours to eliminate residual drug effects. The pharmacokinetic half-life of nicotine in the murine mammal is 1 to 2 hours, which is why vaginal swabs were taken 30 minutes and 1 hour after the final administration of nicotine on day 6.

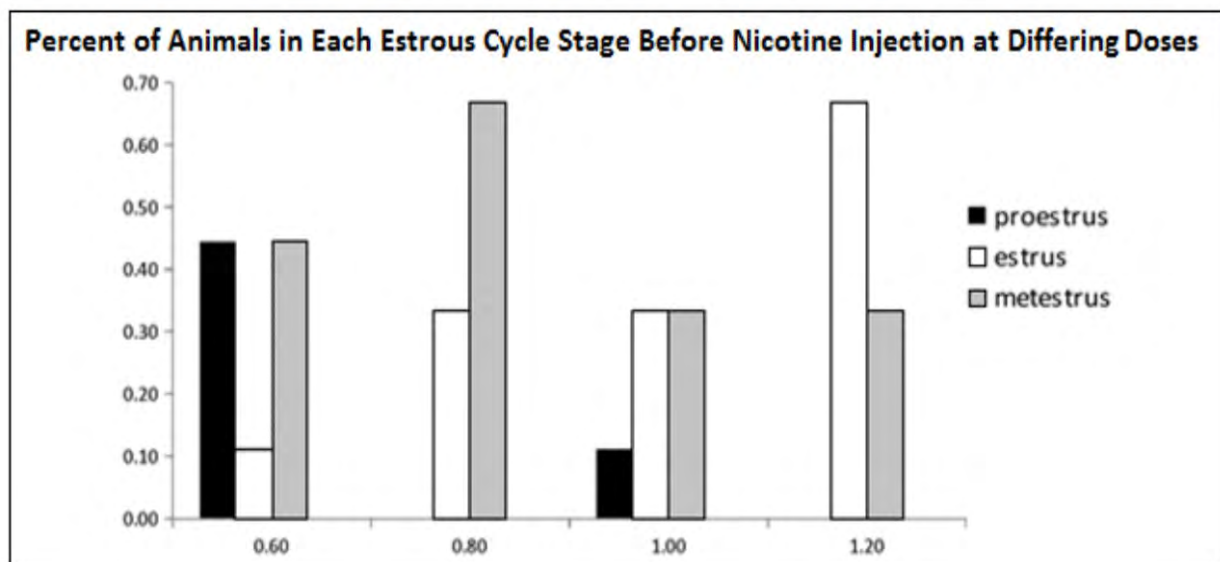
### Vaginal/Exfoliative Cytology

Females were swabbed with a cotton tip soaked in distilled water, and the swabs were dotted on microscope slides. Samples were collected pre-injection, thirty minutes post-6 days of daily nicotine injection, and one hour post-6 days of daily nicotine injection. Diff staining kit (IMEB INC, San Marcos, CA) was used to stain the vaginal smears, and the slides were observed with 20x and 40x objective lenses to accurately determine the cell count per slide. An estimation of the number of anucleated cornified cells, leukocytes, and nucleated epithelial cells was used to determine which stage of the estrous cycle was shown on the slide: proestrus, estrus, metestrus, or diestrus.

## Results

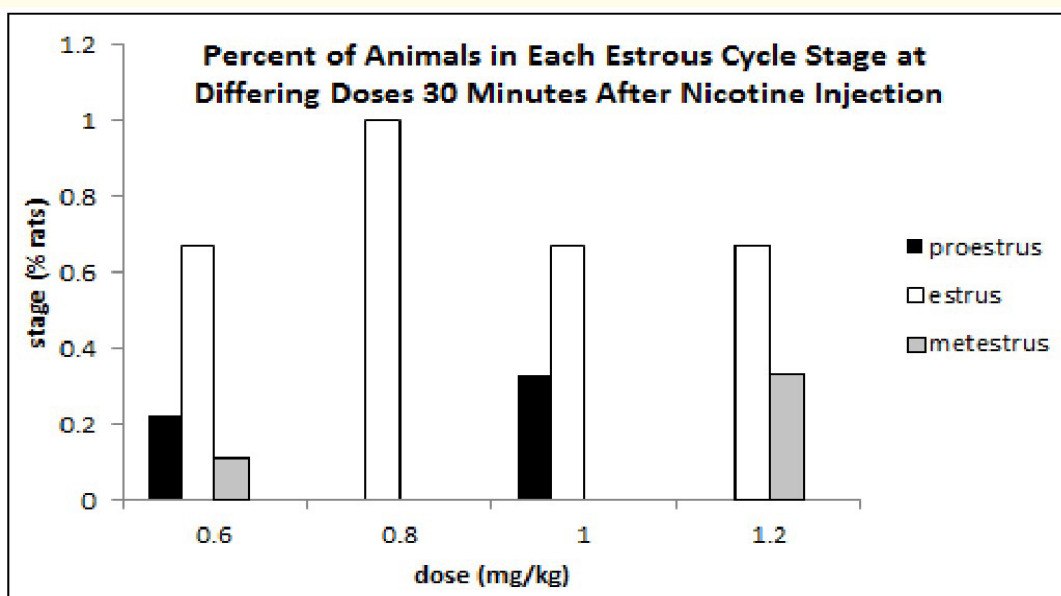
In order to investigate sex differences in nicotine addiction, estrogen and progesterone concentrations were monitored during various stages of the non-prescient mammalian estrous cycle using vaginal swabs taken before, 30 minutes after, and one hour after 6 days of daily nicotine administration at the following dosages: 0.6 mg/kg, 0.8 mg/kg, 1.0 mg/kg, and 1.2 mg/kg. As demonstrated in Figure 4, vaginal smears were used to determine estrous cycle phase by identifying cell types characteristic to each estrous stage. Animals injected with saline instead of nicotine remained in the same stage throughout administration.

Vaginal smears taken before the administration of nicotine demonstrated that the animals were in various stages of the estrous cycle with no significant difference in the number of animals per stage (Figure 4).



**Figure 4:** The percent of animals in each stage of the estrous cycle before nicotine was administered at 4 differing dosages (0.6 mg/kg, 0.8 mg/kg, 1.0 mg/kg, and 1.2 mg/kg). There were no animals in diestrus, and no significant differences in the number of animals in proestrus, estrus, or metestrus.

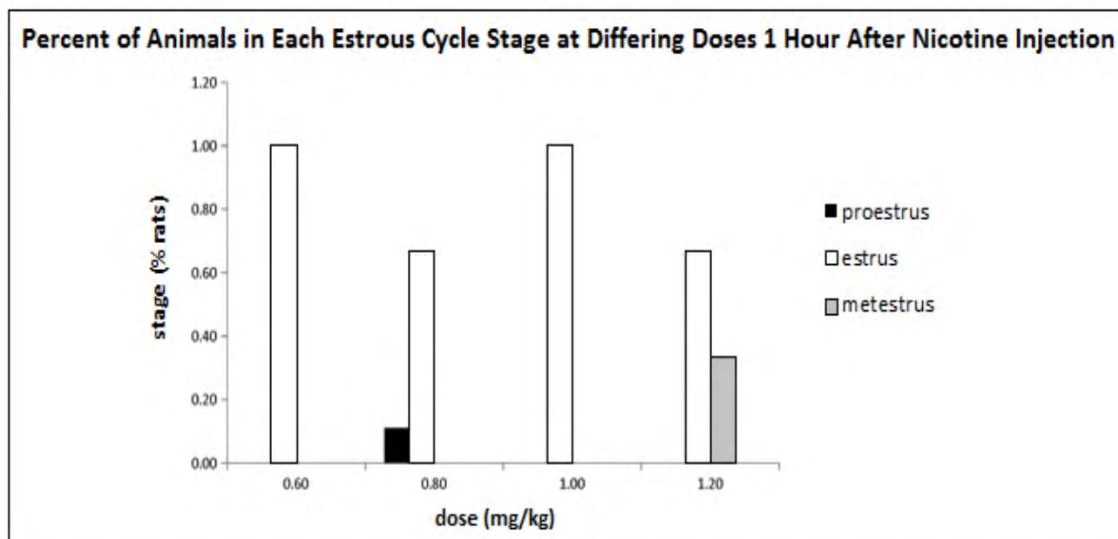
Thirty minutes after 6 days of nicotine administration, a significantly greater number of animals transitioned into estrus from the other stages rather than transitioning into the subsequent stage of the estrous cycle (Figure 5,  $p < 0.001$ ).



**Figure 5:** The total percentage of animals in each stage 30 minutes after 6 days of daily nicotine administration at 4 different dosages (0.6 mg/kg, 0.8 mg/kg, 1.0 mg/kg, and 1.2 mg/kg). There were no animals in diestrus, and the majority of animals were in estrus.

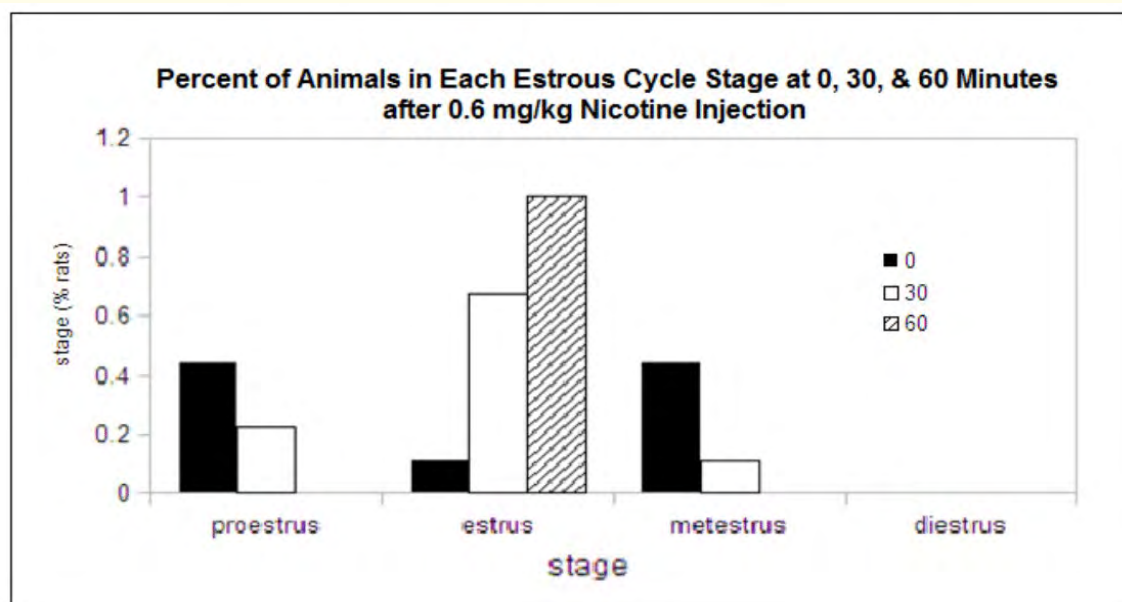
Furthermore, these values remained significant as more animals transitioned into estrus 1 hour after 6 days of daily nicotine administration (Figure 6,  $p < 0.001$ ).





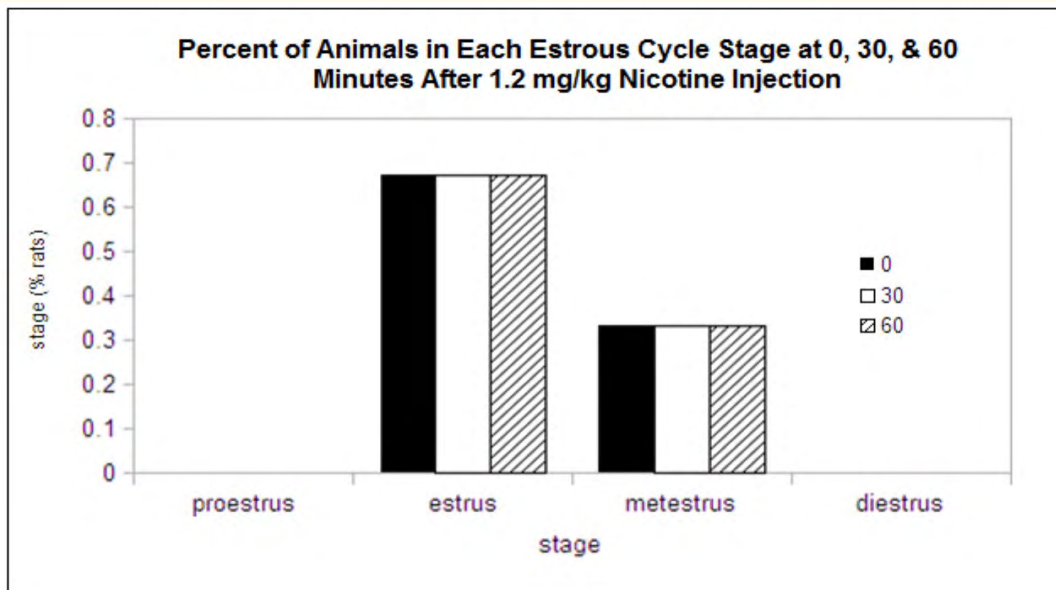
**Figure 6:** The total percentage of animals in each estrous cycle stage 1 hour after nicotine was administered at 4 differing dosages (0.6 mg/kg, 0.8 mg/kg, 1.0 mg/kg, and 1.2 mg/kg) for 6 days. There were no animals in diestrus, and the majority of animals were in estrus.

At the lowest dose of nicotine, 0.6 mg/kg, the majority of animals transitioned to estrus at the end of the 1 hour period following 6 days of daily nicotine administration (Figure 7).



**Figure 7:** The percentage of animals in each stage of the estrous cycle at 0 minutes, 30 minutes, and 60 minutes post-6 days of daily nicotine administration at 0.6 mg/kg.

At the highest dose of nicotine, 1.2 mg/kg, the majority of animals were in estrus at the end of the 1 hour period following 6 days of daily nicotine administration, but no stage changes occurred (Figure 8).



**Figure 8:** The percentage of animals in each stage of the estrous cycle at 0 minutes, 30 minutes, and 60 minutes post-6 days of daily nicotine administration at 1.2 mg/kg. No stage changes were observed at this high dose.

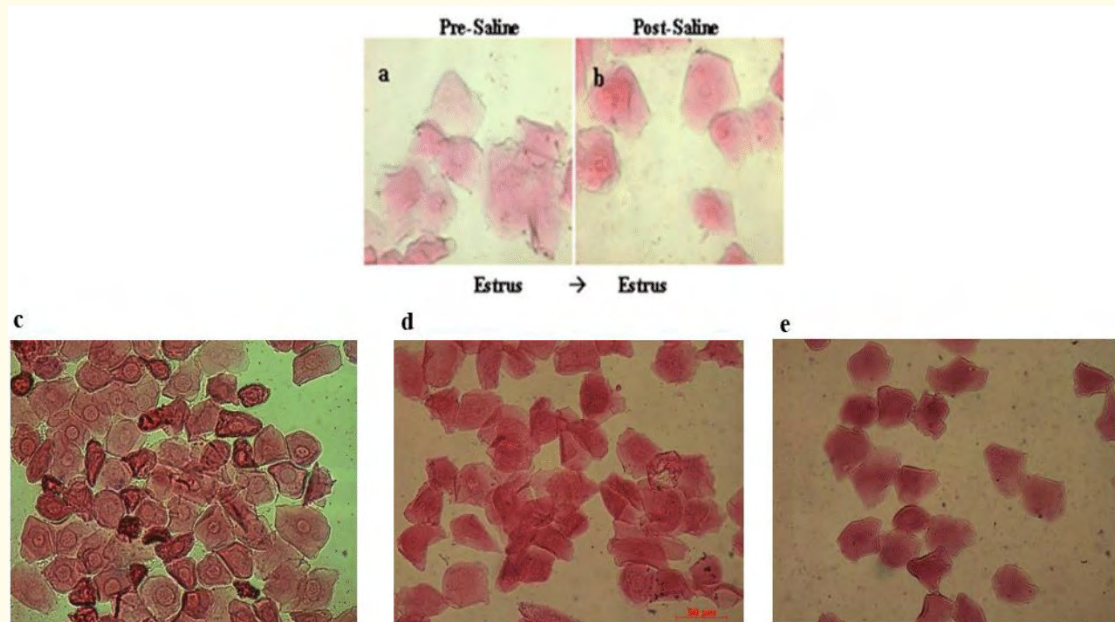
Overall, a significantly greater number of animals were in the estrus stage as compared to other stages of the estrous cycle following nicotine administration at all 4 dosages (0.6 mg/kg, 0.8 mg/kg, 1.0 mg/kg, and 1.2 mg/kg) for 6 days (Table 1).

Stage of Estrous Cycle	Dosage (mg/kg)	Post-Nicotine (Nicotine administration + 30 min)	Recovery (Post-Nicotine + 30 min)
Proestrus (with more anucleated cells)	0.6	2	0
	0.8	0	1
	1.0	1	0
	1.2	0	0
Estrus	0.6	6	9
	0.8	3	2
	1.0	2	3
	1.2	2	2
Metestrus	0.6	1	0
	0.8	0	0
	1.0	0	0
	1.2	1	1
Diestrus	0.6	0	0
	0.8	0	0
	1.0	0	0
	1.2	0	0

**Table 1:** Number of animals in each cycle for 4 different nicotine doses (0.6 mg/kg, 0.8 mg/kg, 1.0 mg/kg, and 1.2 mg/kg) 30 minutes and 1 hour after daily nicotine administration for 6 days.

A significant number of the animals in the proestrus stage transitioned into the estrus stage within 1 hour of nicotine administration. An overwhelming majority of the nucleated epithelial cells became anucleated cornified cells, constituting a stage change from proestrus into estrus (Figure 9).





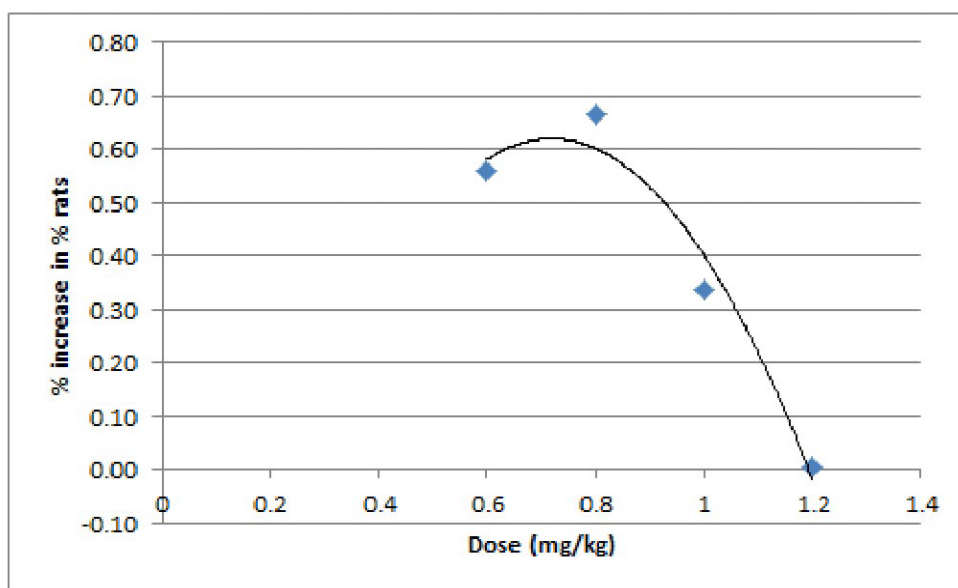
**Figure 9:** a & b.) Control exfoliate vaginal cytology obtained during estrus both before and after saline injection demonstrating a sustained prevalence of nucleated epithelial cells. c.) Pre-nicotine injection: Proestrus demonstrating a disappearance of leukocytes and an increase in both nucleated cornified and non-cornified epithelial cells. d.) Post-6 days of nicotine injection: Estrus phase promotes the appearance of lymphocytes and an increase in anucleated cornified epithelial cells. e.) Recovery: Estrus phase persists.

A small percentage of the animals experienced an increase in a specific cell type before or after a stage change. Of that group, those in the proestrus stage saw an increase in anucleated cells and those in the estrus stage saw an increase in nucleated cells (Table 2); these changes were not statistically significant. Animals in the estrus stage experienced this increase in nucleated epithelial cells after they had transitioned into estrus from another stage. The majority of those in the proestrus stage experienced an increase in anucleated epithelial cells before a transition into another stage. One animal experienced a stage change both before and after the increase in anucleated cells.

Specific Increase in Cell Type	Change in cell type 30 minutes after injection	Change in cell type 1 hour after injection	Stage change occurred before specific increase in cell type	Stage change occurred after specific increase in cell type	No stage change, but increase in cell type occurred
Proestrus with an increase in anucleated cells	3	0	1	3	0
Estrus with an increase in nucleated cells	4	2	5	0	1
Metestrus with more nucleated cells	1	0	0	0	1
Metestrus with more anucleated cells	0	1	0	0	1
Metestrus with both anucleated and nucleated cells	1	1	0	0	2

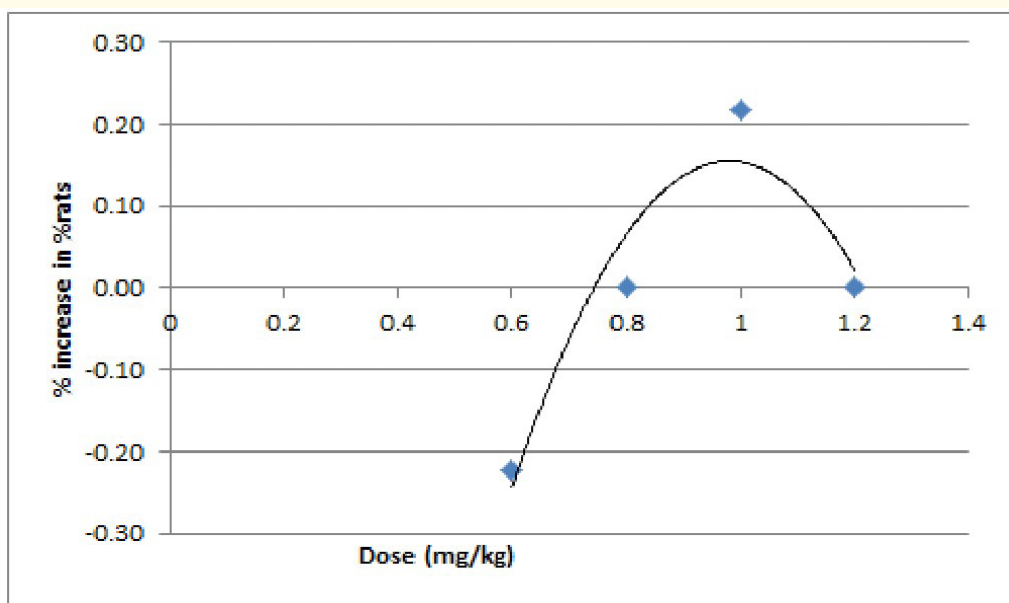
**Table 2:** This table shows the specific increases in cells that were not a statistically significant stage change. The most common increase in specific cell types was an increase in anucleated cells in the proestrus stage and an increase in nucleated cells in the estrus stage.

The percent of animals transitioning into the estrus phase of the estrous cycle peaked at 0.8 mg/kg (Figure 10).



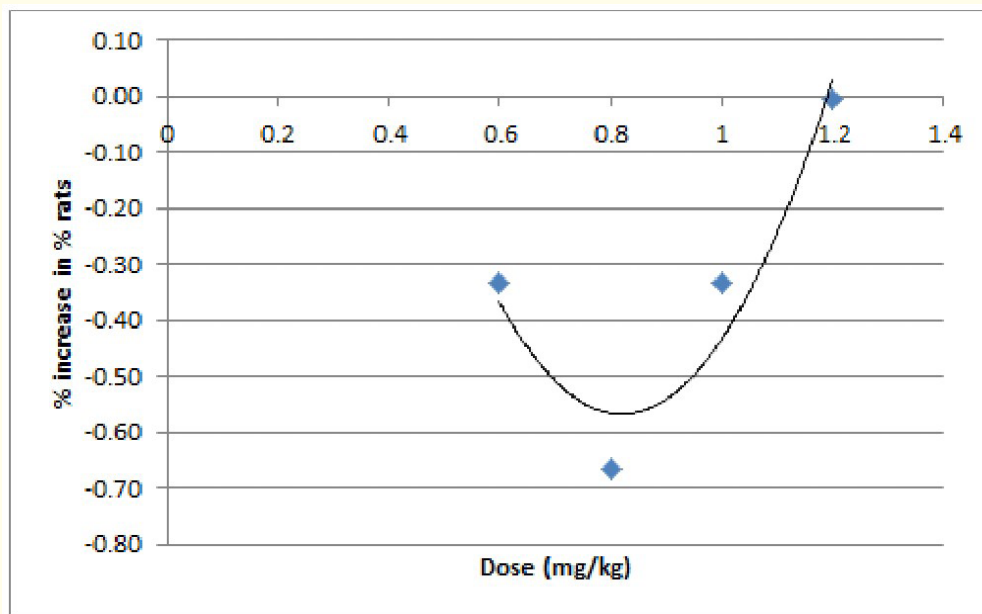
**Figure 10:** Dose response curve for nicotine administration (0.6 mg/kg, 0.8 mg/kg, 1.0 mg/kg, 1.2 mg/kg) and percent increase within the groups of animals in the estrus stage of the cycle 30 minutes after 6 days of daily nicotine administration.

In comparison to Figure 10, the percent of rats in the metestrus stage of the cycle was at its lowest at the 0.8 mg/kg dose of nicotine (Figure 11).



**Figure 11:** Dose response curve for nicotine administration (0.6 mg/kg, 0.8 mg/kg, 1.0 mg/kg, 1.2 mg/kg) and percent increase within the groups of animals in the proestrus stage of the cycle 30 minutes after 6 days of nicotine administration.

The percent increase of animals transitioning into the proestrus stage peaked at 1.0 mg/kg (Figure 12).



**Figure 12:** Dose response curve for nicotine administration (0.6 mg/kg, 0.8 mg/kg, 1.0 mg/kg, 1.2 mg/kg) and percent increase within the groups of animals in the metestrus stage of the cycle 30 minutes after 6 days of nicotine administration.

## Discussion

We hypothesized that nicotine administration would affect the amount of estrogen and progesterone in circulation, thereby affecting estrous cycle phase. For example, increased progesterone would lead to an increase in the number of animals in the proestrus stage of the estrous cycle. This hypothesis was confirmed, and an unexpected trend was identified. As demonstrated by Figures 2 and 3 in conjunction with Table 1, the majority of animals transitioned to the estrus stage 30 minutes and 1 hour after 6 consecutive days of daily nicotine administration. Therefore, nicotine administration is positively correlated with estrus induction in these animals. The induction of estrus was not dose-dependent as demonstrated by the fact that estrus was visualized on vaginal smear regardless of whether nicotine dosage was increased or decreased. Therefore, nicotine administration may perhaps be associated with a decrease in estrogen and progesterone, which resulted in an induction of estrus cycle phase. Due to the fact that nicotine administration was negatively correlated with ovarian hormone concentration (Figures 4, 5, 6), a lack of estrogen and progesterone could be fundamental to understanding the sex differences associated with nicotine addiction. Perhaps a decrease in estrogen and progesterone results in females being more susceptible to the addictive effects of nicotine. Indeed, if nicotine caused ovarian hormones to decrease, then estrogen and progesterone may be protective against nicotine addiction, as well as addiction to other alkaloids such as caffeine and cocaine. Thus, in future research, estrogen and progesterone levels should be measured directly to confirm that nicotine causes a decrease in ovarian hormones.

The pharmacokinetic half-life of nicotine in Sprague Dawley animals is one to two hours, and the pharmacokinetics persist for at least 5 to 7 hours; this is why vaginal swabs were taken 30 minutes and 1 hour after the final administration of nicotine on day 6. At the highest dose of nicotine (1.2 mg/kg), no stage changes were observed (Figure 8). Correspondingly, the dose response curves demonstrate that the percent increase in the number of animals in each stage saw no escalation at the highest dose of nicotine (Figures 10, 11, 12). Due to the fact that the dose response curves reflect approximately 2 weeks of nicotine administration, we hypothesize that the NACHRs became

desensitized to nicotine, resulting in a smaller release of DA in response to nicotine administration. Thus, after receiving the highest dose of nicotine (1.2 mg/kg) for 2 weeks, perhaps the animals required an even higher nicotine dosage to exhibit an estrous cycle stage change via a decrease in ovarian hormones.

The dose response curves further support the conclusion that there is a relationship between nicotine dosage and both estrogen and progesterone concentrations. As demonstrated by Figure 10, the percentage of animals in estrus peaked at 0.8 mg/kg of nicotine. Moreover, the percentage of animals in metestrus reached a nadir at 0.8 mg/kg of nicotine, implying that nicotine impacts the hormones that are most active during the estrus and metestrus stages (Figure 12). The negative curve in Figure 9 demonstrates that the percent of animals in metestrus generally decreased.

In Figure 11, both the dose response curve and the percent of animals transitioning into proestrus peak at 1.0 mg/kg nicotine, which may indicate that the high levels of ovarian hormones associated with proestrus function via a different mechanism during this phase of the estrous cycle.

Table 2 shows specific increases in cell type across the nicotine dosages (0.6 mg/kg, 0.8 mg/kg, 1.0 mg/kg, and 1.2 mg/kg) that were not considered statistically significant enough to be a stage change; this phenomenon is related to the concept of nicotine desensitization in which hormonal concentrations were not affected enough to cause a stage change. Of the animals that showed an increase in a specific type of cell, the majority was in estrus and showed an increase in nucleated cells, which are characteristic to proestrus, 1 hour after 6 days of daily nicotine administration. This increase in nucleated epithelial cells can point to an increase in progesterone and estrogen as both of these hormones peak in proestrus, which is characterized by a predominance of nucleated epithelial cells. This further supports the conclusion that these ovarian hormones play a role in protecting against the effects of nicotine, and that nicotine negatively affects the levels of these hormones. When nicotine leaves the murine system, progesterone and estrogen begin to return to their normal, baseline levels. Additionally, of the animals that showed a specific increase in a particular cell type, the rats in the proestrus stage all showed an increase in anucleated cells, which are characteristic to estrus, 30 minutes after 6 days of daily nicotine administration.

As both estrogen and progesterone levels decrease during the transition from proestrus to estrus, the cells begin to deteriorate and form misshapen, anucleated cornified cells. The non-prescient mammals in proestrus began to show anucleated cells, which signifies a decrease in ovarian hormones. Since this murine cohort may not have experienced a large enough increase in anucleated cells to be considered a stage change from proestrus to estrus, this cellular transformation may be due to the decreased metabolism of nicotine, which would reduce the production of active nicotine metabolites such as cotinine and trans-3'-hydroxycotinine, thereby lowering the amount of active nicotine metabolite to produce an estrous cycle phase change. Moreover, this slower metabolization of nicotine may have occurred under the influence of desensitization. An alternate explanation for this phenomenon is that the cumulative increase in anucleated cells characteristic to estrus was present but was not detected in vaginal swab samples; the anucleated cells may have been below detection threshold due to individual differences regarding the number of cells produced by particular animals. Overall, this increase in anucleated cells delineates the negative direction of the levels of ovarian hormones, and further supports the idea that nicotine causes a decrease in ovarian hormones.

The nicotine hemisulfate salt used in this experiment is a nicotinic acetylcholine receptor agonist. A previous study on estrogen and nicotinic acetylcholine receptors suggested that the estrogen receptor  $\beta$  (ER $\beta$ ) and NACHRs interact in the hypothalamus [39]. ER $\beta$  has been linked to aggression, anxiety, depression, fear, memory, and sexual behavior. An animal model of ER $\beta$ , the murine knockout (KO) model, showed that mice with the KO of ER $\beta$ , also abbreviated as  $\beta$ ERKO, had more regular estrous cycles when compared to non- $\beta$ ERKO mice from the same litter [39]. This implies that ER $\beta$  has a significant impact on changes in the estrous cycle, so it can be concluded that nicotine's interaction with the NACHRs affect the quantity of ER $\beta$ , causing a change in the estrous cycle. However, the action of nicotine as a nicotinic acetylcholine receptor agonist is very complex because it can act on multiple subtypes of AChRs other than ER $\beta$  [40].

### Conclusion

We conclude that nicotine induces estrus in female non-prescient mammals, and that it can affect the amount of both estrogen and progesterone in circulation. Thus, nicotine may have caused a decrease in estrogen and progesterone, which implies that ovarian hormones play a role in protecting against the effects of nicotine. More research should be conducted to understand the mechanism through which NACHRs interact with estrogen and progesterone via neurotransmission. Moreover, further research examining the relationship between DA and the sex hormones should be conducted. Prior studies on ovariectomized, non-prescient mammals submitted to 2 days of estradiol injections with a co-injection of estradiol and progesterone on day 3 demonstrated that estrogen and progesterone may cause inhibition with a subsequent growth in the density of striatal DA receptors [41]. However, a more detailed understanding of the interaction between DA, estrogen, and progesterone may elucidate the sex differences of addiction to alkaloids such as nicotine.

Finally, based on feedback received at a presentation of this work at the 2016 Society for Neuroscience Conference [33], further research should replicate this study and take vaginal swabs hours to days after the final dose of nicotine on day 6. In this manner, the longevity of nicotine's ability to induce estrus may be evaluated. Moreover, future research may examine whether different routes of administration may alter the ability of nicotine to change estrus cycle phase. Finally, future studies may be designed to determine the minimum nicotine dose necessary to artificially induce estrus. Indeed, examining the interaction between addictive alkaloids and sex hormones may be fundamental to tailoring therapies for female substance abuse and addiction, as well as understanding the implications of alkaloid addiction on initiating or sustaining a pregnancy.

### Acknowledgements

The authors wish to thank the Broderick Brain Foundation, the F.M. Kirby Foundation, the Center for Advanced Technology, CUNY, and the MacKenzie Foundation for partial support of our laboratory and students during these studies. It is important to note that since this body of work, the development and pioneering of Neuromolecular Imaging and the BRODERICK PROBE® has taken place diligently for many years. Other grants including the National Institute of Health, National Institute on Drug Abuse, The Lowenstein Foundation, the FACES and PACE Foundations for Epilepsy and The Upjohn Pharmacia Company in Michigan deserve honorable mention. Finally, the authors wish to announce the recent May 3, 2017 contractual agreement between Eazysense Nanotechnologies, Inc. and Indian Angel Network®, to advance the marketing of the BRODERICK PROBE®.

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