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# Examination Of Sex Differences And The Role Of Ovarian Hormones In Modulating Nicotine Withdrawal In Rats

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# EXAMINATION OF SEX DIFFERENCES AND THE ROLE OF OVARIAN HORMONES IN MODULATING NICOTINE WITHDRAWAL IN RATS

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Lovingly dedicated to my beautiful wife, Monica Flores, for all the love and support she has provided me with throughout graduate school.

# EXAMINATION OF SEX DIFFERENCES AND THE ROLE OF OVARIAN HORMONES IN MODULATING NICOTINE WITHDRAWAL IN RATS

By

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## Abstract

*Introduction*: **Aim 1** characterized sex differences and the role of ovarian hormones in physical signs and negative affective states produced by nicotine withdrawal in female, ovariectomized female (OVX), and male rats. We also compared nicotine withdrawal and corticosterone levels with estradiol (E2) and progesterone across the 4-day estrous cycle. **Aim 2** validated the role of ovarian hormones in withdrawal in OVX females that received vehicle, E2, or E2+progesterone. *Methods***:** Female rats received a sham surgery or an ovariectomy procedure. Fifteen days later, all rats were implanted with a pump that delivered nicotine for 14 days. On the test day, separate groups received saline or mecamylamine to precipitate withdrawal. Rats were then examined for physical signs of withdrawal and anxiety-like behavior. After testing, serum levels of corticosterone, E2, and progesterone were assessed. Female rats received vaginal lavage procedures to verify the phase of the estrous cycle on the test day. *Results:* Intact females displayed greater anxiety-like behavior and higher corticosterone during withdrawal as compared to males and OVX females. Intact females that were tested in estrus (when E2 levels are relatively low) displayed less anxiety-like behavior and corticosterone as compared to all other phases. During withdrawal, anxiety-like behavior and corticosterone were positively correlated with E2 and negatively correlated with progesterone and the progesterone/E2 ratio. Intact females displaying high E2/low progesterone levels displayed greater anxiety-like behavior and corticosterone as compared to females displaying low E2/high progesterone levels. Lastly, OVX rats that received E2 displayed greater anxiety-like behavior as compared to OVX rats that received vehicle. OVX rats that received E2+progesterone displayed less anxiety-like behavior as compared to OVX rats that received E2 alone. *Conclusion*: These data suggest that E2 promotes, whereas progesterone reduces anxiety-like behavior produced by nicotine withdrawal.

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# Chapter 1: Introduction

## 1.1 Tobacco use is a public health problem

Tobacco use is the leading cause of preventable deaths in the United States, with over 400,000 deaths per year attributed to smoking-related causes (U.S. Department of Health and Human Services, 2014). Long-term tobacco use is associated with adverse health consequences such as lung cancer, cardiovascular disease, and emphysema (D'Alessandro et al., 2012; Hecht, 2012; Milara and Cortijo, 2012). In addition to being a significant public health problem, tobacco use is also an economic burden. Indeed, the costs associated with tobacco use exceeds more than 300 billion dollars each year (U.S. Department of Health and Human Services, 2016). Given the magnitude of problems associated with tobacco use, more research is needed to understand the mechanisms underlying smoking behavior.

## 1.2 Tobacco use is driven by nicotine reward and avoiding withdrawal

Studies have identified nonpharmacological factors that motivate tobacco use in humans such as the need for appetite suppression, the desire to improve self-image, peer-pressure, and anxiety management (Seguire & Chalmers 2000; Reid et al., 2009; Perkins et al., 1997;1999 & 2001). However, much work has also revealed that individuals use tobacco to experience the psychopharmacological effects of nicotine, the main habit-forming compound in tobacco (U.S. Department of Health and Human Services, 2014; Pogocki et al., 2007; Pontieri et al., 1996). Current theories suggest that both positive and negative reinforcement processes motivate tobacco use (George and Koob, 2017). For example, initial tobacco use is thought to be driven primarily by nicotine acting as a positive reinforcer. Specifically, nicotine produces euphoria, increases energy, and enhances attentional processes (Benowitz, 1996; Kaye et al., 2014; Pomerleau & Pomerlaeau, 1992). Following chronic exposure, tobacco use is believed to be driven primarily via

negative reinforcement involving the avoidance of negative affective states produced by nicotine withdrawal (George and Koob, 2017; Pipkin et al., 2017). Indeed, abstinence from long-term tobacco use leads to a withdrawal syndrome consisting of negative physical and affective states that are alleviated by subsequent drug taking. The physical symptoms include nausea, headaches, sleep disturbances, and an increase in food cravings (Hogle et al., 2006; Shiffman et al., 2004; Hughes, 2007). The negative affective states produced by nicotine withdrawal include intense craving, irritability, anxiety, depression, difficulty concentrating, as well as other forms of cognitive impairments (Hughes et al., 1992; Pauly, 2008; Perkins et al., 2009; Hatsukami et al., 1989 and Heishma et al., 1994). The emergence of physical and negative affective states during abstinence are believed to promote continued tobacco use and relapse, as tobacco users will smoke to avoid experiencing the symptoms of nicotine withdrawal (Baker et al., 2004; Battista et al., 2008; Fidler et al., 2009; Hughes, 2007; Piper et al., 2011; Ríos-Bedoya et al., 2008).

**1.3 Women are more vulnerable to tobacco use than men**

Clinical reports suggest that women are more vulnerable to tobacco use than men. Although men consume more tobacco products (Jamal et al., 2015), women are at higher risk of developing tobacco-related diseases, including cancer and chronic obstructive pulmonary disease (Kiyohara and Ono 2010; Langhammer et al., 2000). Women also exhibit lower smoking cessation rates and are less likely to benefit from nicotine replacement therapy (NTR) than men (Cepeda-Benito et al., 2004; Perkins 2001; Perkins and Scott, 2008; Piper et al., 2010). Women also display stronger negative affective states during abstinence than men (Panagiotakopoulos and Neigh, 2014; Perkins et al., 2012a, 2012b; Weinberger and McKee, 2012), and they report higher levels of depression, anxiety, and intense craving during smoking abstinence as compared to men (al'Absi, 2006; Schnoll et al., 2007; Xu et al., 2008; Leventhal et al., 2007; Pang et al., 2018).

Consistent with the latter reports, women display higher levels of cortisol during smoking abstinence than men (Hogle and Curtin, 2006). Not surprising, women report more often than men that they smoke to avoid negative affective states produced by nicotine withdrawal (Stanton, 1995). These studies suggest that withdrawal from chronic tobacco use leads to higher levels of anxiety and biological stress responses in women that likely promote higher rates of smoking relapse in women as compared to men.

**1.4 Ovarian hormones and tobacco use in women**

A growing body of literature suggests that the ovarian hormones, estradiol (E2) and progesterone modulate sex differences in the positive subjective effects of nicotine. One approach to examine the relationship between ovarian hormones and the effects of drugs of abuse in women is to examine responses to drugs of abuse across the different phases of the menstrual cycle (Anker & Carrol, 2010). In women, the menstrual cycle is divided into two phases: the follicular stage, where E2 levels are rising relative to progesterone, and the luteal phase where progesterone levels are high relative to E2 (Owen, 1975). Women report greater subjective effects of nicotine in the follicular versus the luteal phase of the menstrual cycle (DeVito et al., 2014). These findings are consistent with work showing that women display greater positive subjective responses to cocaine and amphetamine during the follicular as compared to the luteal phase of the menstrual cycle (Evans and Foltin, 2006; Evans et al., 2002; Sofuoglu et al., 1999; Justice and de Wit, 1999). Given the hormonal profile of the follicular (high E2) and luteal (high progesterone) phases, one might expect that E2 promotes and progesterone attenuates the positive subjective effects of nicotine (Sofuoglu et al., 2001, 2011).

Clinical reports also suggest that ovarian hormone fluctuations influence negative affective states produced by nicotine withdrawal. Specifically, women who quit in the luteal phase of their

menstrual cycle experience more withdrawal signs as compared to women that quit in the follicular phase (O'Hara et al., 1989; Perkins et al., 2000). A meta-analytic review also revealed that women reported greater withdrawal during the luteal versus follicular phase (Weinberger et al., 2015). It is important to note that other reports have not found differences in the severity of the withdrawal syndrome in women who quit during the follicular versus the luteal phase of the menstrual cycle (Allen et al., 2000 & 2010). Although these studies suggest that ovarian hormones modulate nicotine withdrawal in women, the relationship between withdrawal severity and E2 and progesterone levels remains unclear. This question requires pre-clinical rodent studies that can study nicotine withdrawal while manipulating ovarian hormones levels. Thus, this dissertation examined the role of E2 and progesterone in a rodent model of nicotine withdrawal.

**1.5 Female rat estrous cycle**

In contrast to humans that display a 28-day menstrual cycle, female rats display a 4-5 day estrous cycle. The rodent estrous cycle is similar to the human menstrual cycle that is tightly regulated by circulating levels of E2 and progesterone, with receptors for these hormones in the brain of both rats and humans (Schedin et al., 2000). The estrous cycle consists of 4 phases: proestrus, estrus, metestrus, and diestrus. The proestrus stage is characterized by peak increases in E2 and progesterone. The estrus stage is characterized by a steep decline in E2 and a gentle decline in progesterone levels. The metestrus phase is characterized by stable low levels of E2 and a gradual increase in progesterone. Lastly, the diestrus stage is characterized by stable low levels of both E2 and progesterone (Butcher et al., 1974; Haim et al., 2003). The effects of ovarian hormones on a given behavioral outcome can be examined following the removal of the ovaries and subsequent E2 or progesterone supplementation.

### 1.6 Sex differences in rodent models of nicotine withdrawal

In rodents, nicotine dependence can be induced via surgical implantation of an osmotic pump that delivers nicotine for 7-14 days (Kenny and Markou, 2001; Malin, 2001; O'Dell et al., 2004). Nicotine withdrawal can then be assessed following removal of the pump (spontaneous withdrawal) or following administration of a nicotinic receptor antagonist, such as mecamylamine (precipitated withdrawal). By either method, nicotine withdrawal produces a behavioral profile in rodents that is comprised of physical signs and negative affective states. The physical signs of nicotine withdrawal include facial fasciculations, teeth chatters, writhes, gasps, eye blinks, and ptosis (Malin, 2001; O'Dell et al., 2004; Watkins et al., 2000). The negative affective states elicited by withdrawal include anxiety-like behavior that can be assessed as an decrease in time spent in the open arms of an elevated plus maze (EPM; Bruijnzeel et al., 2012; Tejeda et al., 2012; Wilmouth and Spear, 2006) or the lit compartment of a light-dark transfer (LDT) apparatus (Stoker et al., 2008) relative to control animals. This dissertation employs both EPM and LDT tests to provide converging lines of evidence of anxiety-like behavior produced by withdrawal.

Previous studies have revealed that there are sex differences in the magnitude of negative affective states produced by nicotine withdrawal in rodents. Specifically, females display a larger aversion to an environment paired previously with nicotine withdrawal as compared to male rats (O'Dell and Torres, 2014) and mice (Kota et al., 2007, 2008). Female rats also display higher serum levels of corticosterone and adrenocorticotropic hormone during nicotine withdrawal as compared to males (Gentile et al., 2011; Skwara et al., 2012). During nicotine withdrawal, female rats also display higher levels of anxiety-like behavior and expression of stress-associated genes in the nucleus accumbens (NAc) than males (Torres et al., 2013). These studies suggest that female rats experience greater stress during nicotine withdrawal as compared to males. Previous work has

also revealed that OVX rats display reduced negative affective states during nicotine withdrawal as compared to intact female rats (Torres et al., 2015). OVX rats also display a reduction in corticosterone levels and decreased expression of stress-associated genes in the NAc relative to intact females (Torres et al., 2015). Previous work has also revealed that OVX females display less nicotine intravenous self-administration as compared to intact females, an effect that was reversed in OVX females that received E2 supplementation (Flores et al., 2016). Taken together, these studies suggest that the behavioral effects of nicotine are ovarian hormone-dependent. However, the specific role of E2 and progesterone in promoting nicotine withdrawal have not been examined.

### 1.7 Critical knowledge gap this dissertation addresses

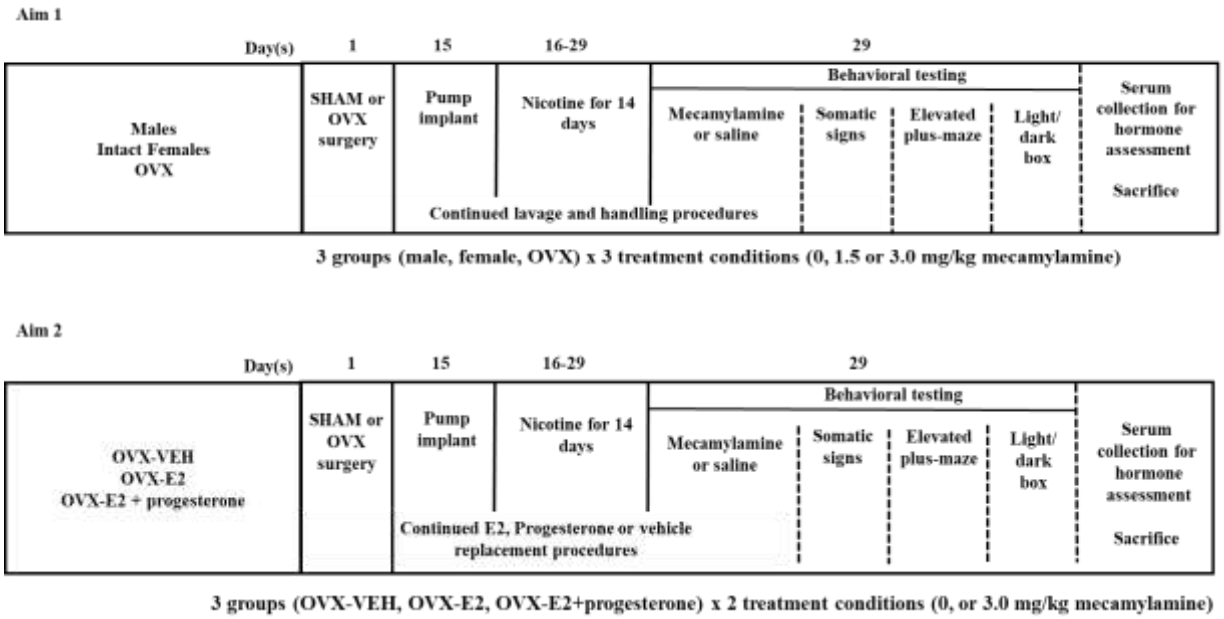
Clinical evidence suggests that the stress produced by nicotine withdrawal drives smoking behavior and relapse during nicotine withdrawal. However, there is a lack of pre-clinical studies examining sex differences in the stress response produced by withdrawal. Furthermore, the role of E2 and progesterone in promoting stress during withdrawal remains unclear. This dissertation addressed these issues by examining behavioral and biological indices of stress produced by nicotine withdrawal in rats. **Aim 1** characterized sex differences and the role of ovarian hormones in nicotine withdrawal. This was achieved by assessing nicotine withdrawal in male, intact female, and OVX rats. Next, the magnitude of withdrawal was compared in intact females tested in proestrus, estrus, metestrus, and diestrus. Lastly, the relationship between withdrawal and levels of E2 and progesterone were assessed in intact females. **Aim 2** validated the role of ovarian hormones in nicotine withdrawal. This was achieved by examining whether ovarian hormone replacement influenced the expression of nicotine withdrawal in OVX rats that received vehicle, E2, or E2+progesterone supplementation.

## Chapter 2: Methods

### 2.1 Subjects

Male and female Wistar rats were obtained from an out-bred stock of animals (Envigo, Inc., Indianapolis, IN). On post-natal day (PND) 21, the rat pups were weaned and housed with a same-sex littermate for the remainder of the study. The rats were housed in a humidity- and temperature-controlled (22˚C) vivarium on a reverse 12-hour light/dark cycle (lights off at 8:00 am and on at 8:00 pm) and ad libitum access to food and water. Prior to beginning the experiment, the rats were handled for 5 days. All the experimental procedures were approved by the UTEP Institutional Animal Care and Use Committee.

### 2.2 Overall design




These diagrams depict our experimental groups and procedures. Aim 1 compared sex differences and the role of ovarian hormones in the behavioral effects of nicotine withdrawal. Nicotine-dependent male, intact female, and OVX female rats received saline or mecamylamine (1.5 or 3.0 mg/kg) to precipitate withdrawal on the test day. Aim 2 examined the role of E2 and

E2+progesterone in the behavioral effects of nicotine withdrawal. Nicotine-dependent OVX females were supplemented with vehicle, E2, or E2+progesterone, and they received saline or mecamylamine (3.0 mg/kg) on the test day. Only the highest dose of mecamylamine was included because sex differences in nicotine withdrawal were observed at this dose and robust withdrawal effects were only detected at this high dose in OVX females in Aim 1. The rats received either a surgical sham or OVX procedure. Fifteen days later, rats were prepared with an osmotic pump that delivered nicotine continuously. Following 14 days of nicotine exposure, the rats received mecamylamine to precipitate withdrawal and they were tested in a series of behavioral tests that included the physical signs and two tests of anxiety-like behavior, the EPM and LDT procedures. At the end of testing in Aim 1, trunk blood was collected to assess serum E2 and progesterone levels. Also, the intact females received vaginal lavage procedures to assess the phase of the estrous cycle that they were tested in. After behavioral testing in each study, trunk blood was collected to assess serum corticosterone levels in all groups.

**2.3 Surgery**

At PND 45-46, the rats were anesthetized using an isoflurane/oxygen mixture (1-3% isoflurane). Some of the female rats received surgical removal of ovarian tissue, as previously described (Torres et al., 2009). In order to control for any behavioral disruptions produced by surgery, male and intact female rats received a sham procedure consisting of anesthesia and then an incision in the same location as the OVX procedure. The OVX was done at PND 45-46 based on previous work in our laboratory showing that female rats that received OVX procedures at PND 45 display a reduction in the rewarding effects of nicotine (Torres et al., 2009; Flores et al., 2016) and a suppression of anxiety-like behavior and stress-associated gene expression during nicotine withdrawal (Torres et al., 2013, 2015). These studies suggest that after PND 45, ovarian hormones

play a key role in modulating the behavioral effects of nicotine withdrawal. Once the rats reached PND 60, the rats were re-anesthetized and then surgically prepared with an osmotic pump that delivered nicotine continuously for 14 days (3.2 mg/kg/day, expressed as base; model 2ML2; 5.0 ul/hour; Durect Corporation, Inc.). Previous studies in our laboratory have shown that this dose of nicotine produces similar levels of cotinine (a major nicotine metabolite) in male and female rats (O'Dell et al., 2007).

**2.4 Behavioral testing**

The rats were removed from the vivarium and placed into a clear Plexiglas® cage in a test room that was dedicated to the assessments of physical signs of withdrawal under regular light conditions. Following a 10-min acclimation period, the rats received an injection of saline or mecamylamine (1.5 or 3.0 mg/kg), and 10 min later the physical signs of withdrawal were assessed. The observed signs included blinks, writhes, body shakes, teeth chatters, gasps, grooming bouts, licks, and ptosis. Multiple successive counts of any sign required a distinct pause between episodes. Ptosis was only counted once per min. The total number of somatic signs were defined as the sum of individual occurrences of the signs during the entire observation period. Following the physical signs assessment, the rats were placed in a transport cage and acclimated to another dimly lit room for 5 min prior to testing in the EPM. The EPM apparatus consists of 4 arms elevated to a height of 50 cm above the ground. The apparatus was illuminated by a red light suspended from the ceiling of a dark room. At the beginning of the test, the rats were placed in the center of the EPM facing an open arm. Time spent in the center area, open, and closed arms were recorded for 5 min. Anxiety-like behavior was operationally defined as a decrease in time spent in the open arms relative to control rats. Following EPM testing, the rats were returned to their transport cage and acclimated for 5 min to a separate room dedicated to the LDT test. The LDT

apparatus consists of 2 chambers, one side consists of clear Plexiglas® and the other side is enclosed and painted solid black. The apparatus is separated by a wall with an opening that allowed the rats to pass between the 2 sides freely. The apparatus was positioned in the middle of the room under regular light conditions. At the start of the test, the rats were placed in the middle of the dark chamber facing the back wall of the apparatus. The time spent in each side was scored for 5 min. Anxiety-like behavior test was operationally defined as a decrease in time spent in the lit compartment relative to control rats. The test equipment was thoroughly cleaned and dried before each rat was tested.

**2.5 Hormone level assessments**

After behavioral testing, the intact female rats in Aim 1 were sacrificed, and trunk blood was collected. The blood was centrifuged for 15 min at 5000 x g at 4°C. Serum was extracted and stored in 100 ul aliquots at -80°C until analyzed via enzyme-linked immunosorbent assay (ELISA) procedures for progesterone (Enzo Life Sciences, Farmingdale, USA) and corticosterone (Assaypro, Winfield, MO) according to the manufacturer instructions. The standards ranged from 0 to 100 ng/mL for corticosterone and 0 to 500 pg/mL for progesterone, and they were included in every assay. Samples were placed in a 96 well-plate and read at 450 and 630 nm wavelength using a Spectra Maxplus spectrophotometer (Molecular Devices, Inc.). E2 levels were assayed at the University of Pittsburgh Small Molecule Biomarker Core, using liquid chromatography-tandem mass spectrometry (LC-MS/MS). LC-MS/MS is the preferred method to estimate serum E2 levels given that the serum concentration of this hormone is below a detectable range of sensitivity for standard immunoassays, such as ELISA.

**2.6 Estrous cycle determination**

Vaginal lavage procedures were used to estimate the phase of the estrous cycle (i.e., proestrus, estrus, metestrus, or diestrus). In Aim 1, the lavage procedures began 8 days prior to the pump surgery and continued until the end of the final test day. As a control procedure, the male rats were handled every day. In Aim 2, OVX females only received lavage procedures on the final test day. A sterile and disposable plastic pipette was filled with 0.9% saline and was used to collect epithelial cells. Epithelial cells were then transferred to a labeled glass microscope slide. Microscope slides were fixed with methylene blue stain (Sigma, Inc.) and viewed under a light microscope at 40X to examine the shape of the cells and determine the phase of the estrous cycle by the following criteria: proestrus=presence of round nucleated epithelium cells, estrus=presence of cornified un-nucleated epithelium cells, metestrus=limited presence of epithelium cell and leukocytes and diestrus=presence of leukocytes (Goldman et al 2007).

**2.7 Hormone supplementation procedures**

In Aim 2, OVX females received a 4-day E2 supplementation procedure that began the following day after ovariectomy surgery. OVX controls received repeated vehicle injections (peanut oil). To examine the effects of E2 alone, a group of OVX females received 2 days of a 0.1 mL bolus injection of E2 (5 µg), 1 day of vehicle injection, and no injection on the final day of the 4-day cycle. This supplementation procedure mimics normal E2 cycling patterns in intact female rats (see Asarian et al., 2002). On the test day, the rats received their E2 injection 30 min prior to the beginning of the behavioral battery. To examine the effects of E2 and progesterone, a group of OVX females received 2 days of a 0.1 mL bolus injection of E2 (5 µg), 1 day of progesterone (250 µg), and no injection on the final day of the 4-day cycle. On the test day, rats received progesterone 4.5 hours prior to the beginning of the behavioral battery. The 4-day supplementation procedures

were repeated 5 times prior to testing. A group that received progesterone alone was not included because progesterone only induces sexual receptivity in OVX rats that are supplemented with E2+progesterone (Becker et al., 2005).

**2.8 Statistics**

In Aim 1, the data were analyzed separately for each measure (physical signs, EPM, LDT, and corticosterone) using 2-way ANOVAs with group (male, female, and OVX) and treatment condition (0, 1.5, and 3.0 mg/kg of mecamylamine) as between subject factors. Across all measures, significant interaction effects were further analyzed using post-hoc comparisons where appropriate. To examine estrous cycle effects, the female rats that received the 3.0 mg/kg dose of mecamylamine were grouped according to the stage of estrous they were tested in. The data were then analyzed separately for each measure (physical signs, EPM, LDT, and corticosterone) using a 1-way ANOVA with stage of estrous (proestrus, estrus, metestrus and diestrus) as a between subjects factor. The relationship between hormones and the behavioral effects of withdrawal was examined via simple linear regression. Each hormone was correlated separately and as a ratio of progesterone to E2 with each measure of nicotine withdrawal. The ratio of these hormones was compared based on prior work showing that influence of ovarian hormones on nicotine IVSA is most evident when E2 and progesterone levels are expressed in relative proportion to each other (see Lynch, 2009). The measures of nicotine withdrawal were then regressed on the progesterone/E2 ratios and the Pearson correlation co-efficient was computed to assess the strength and direction of each relationship. A one-sample t-test was used to examine whether the correlations were statistically significant. In intact female rats, the relative concentration of E2 and progesterone varies during each phase of the estrous cycle. We used statistical approaches to assess whether intact females could be categorized into groups that displayed similar concentrations of

these hormones. A k-means clustering algorithm was used to classify rats according to their levels of E2 and progesterone. This analysis resulted in 2 groups that had significantly different hormone levels (high E2/low progesterone and low E2/high progesterone). Individual rats were grouped according to their group membership and differences in each measure (physical signs, EPM, LDT, and corticosterone) were compared using a between-subjects t-test. This k-means categorization process has been used to classify older and younger animals according to their performance in working memory and behavioral flexibility tasks (Mota et al., 2019). In Aim 2, the data were analyzed separately for each measure (physical signs, EPM, LDT, and corticosterone) using 2-way ANOVA with group (OVX-VEH, OVX-E2, OVX-E2+progesterone) and treatment (0 or 3.0 mg/kg of mecamylamine) as between subject factors. Significant interaction effects were further analyzed using post-hoc comparisons where appropriate.

## Chapter 3: Results

### 3.1 Aim 1

*Figure 1* displays physical signs (A), EPM (B), LDT (C), and corticosterone levels (D) in nicotine-treated male, intact female, and OVX rats that received mecamylamine (0, 1.5, or 3.0 mg/kg) on the test day. With regard to physical signs, there were no interaction effects between group and treatment conditions [$F(4,154)=0.57$; $p=0.58$]. However, there was a main effect of treatment condition [$F(2,154)=98.33$; $p\leq0.05$], with rats that received the 1.5 or 3.0 mg/kg dose of mecamylamine displaying more physical signs as compared to saline controls (*$p\leq0.05$).

With regard to the EPM data, a 2-way ANOVA of percent time in open arms revealed a significant interaction between group and treatment condition [$F(4,154)= 2.87$; $p\leq0.05$]. In males, post-hoc analyses revealed a significant decrease in % time in open arms in rats that received the 1.5 or 3.0 mg/kg dose of mecamylamine as compared to saline controls (*$p\leq0.05$). There was no difference between males that received the 1.5 or 3.0 mg/kg dose of mecamylamine ($p=0.47$). In intact females, post-hoc analyses revealed a significant decrease in % time in open arms in rats that received the 1.5 or 3.0 mg/kg dose of mecamylamine as compared to saline controls (*$p\leq0.05$). The % time in open arms was lower in intact females that received the 3.0 versus 1.5 mg/kg dose of mecamylamine (#$p\leq0.05$). In OVX females, post-hoc analyses revealed a significant decrease in % time in open arms in rats that received the 1.5 or 3.0 mg/kg dose of mecamylamine as compared to their respective saline controls (*$p\leq0.05$). There was no difference between OVX rats that received the 1.5 versus 3.0 mg/kg dose of mecamylamine (*$p=0.59$). Group differences were detected in rats that were treated with the 3.0 mg/kg dose of mecamylamine. Post-hoc analyses revealed that % time in open arms was lower in intact females as compared to male and OVX rats (†$p\leq0.05$).

With regard to the LDT data, a 2-way ANOVA of percent time the lit compartment revealed a significant interaction between group and treatment condition [$F(4,154)=3.02$; $p\leq0.05$]. In males, post-hoc analyses revealed a significant decrease in % time in the lit compartment in rats that received the 3.0 (*$p\leq0.05$), but not the 1.5 ($p=0.06$) mg/kg dose of mecamylamine as compared to saline controls. There was no difference between males that received the 1.5 or 3.0 mg/kg dose of mecamylamine ($p=0.25$). In intact females, post-hoc analyses revealed a significant decrease in % time in the lit compartment in rats that received the 1.5 or 3.0 mg/kg dose of mecamylamine as compared to female saline controls (*$p\leq0.05$). The % time in the lit compartment was lower in intact females that received the 3.0 versus 1.5 dose mg/kg of mecamylamine (#$p\leq0.05$). In OVX females, post-hoc analyses revealed a significant decrease in % time in the lit compartment in rats that received the 3.0 (*$p\leq0.05$), but not the 1.5 ($p=0.28$) mg/kg dose of mecamylamine as compared to OVX saline controls. The % time in the lit compartment was lower in OVX females that received the 3.0 versus 1.5 mg/kg dose of mecamylamine (#$p\leq0.05$). Group differences were detected in rats that were treated with the 1.5 mg/kg dose of mecamylamine. Post-hoc analyses revealed that % time in the lit compartment was lower in intact females as compared to OVX rats (@$p\leq0.05$). Group differences were also detected in rats that were treated with the 3.0 mg/kg dose of mecamylamine. Post-hoc analyses revealed that % time in the lit compartment was lower in intact females as compared to male and OVX rats (†$p\leq0.05$).

With regard to corticosterone levels, a 2-way ANOVA revealed a significant interaction between group and treatment condition [$F(4,154)=2.60$; $p\leq0.05$]. In males, post-hoc analyses revealed a significant decrease in % time in the lit compartment in rats that received the 1.5 or 3.0 mg/kg dose of mecamylamine as compared to saline controls (*$p\leq0.05$). There was no difference

between males that received the 1.5 or 3.0 mg/kg dose of mecamylamine ($p$=0.18). In intact females, post-hoc analyses revealed a significant increase in corticosterone in rats that were treated with the 1.5 or 3.0 mg/kg dose of mecamylamine as compared to saline controls (*$p \leq 0.05$). Corticosterone was also higher in intact females that received the 3.0 versus 1.5 mg/kg dose of mecamylamine ($p \leq 0.05$). In OVX females, post-hoc analyses revealed a significant increase in corticosterone in rats that were treated with the 3.0 (*$p \leq 0.05$), but not the 1.5 ($p$=0.59) mg/kg dose of mecamylamine as compared to saline controls. Corticosterone did not differ in OVX rats that received the 1.5 versus the 3.0 mg/kg dose of mecamylamine ($p$=0.34). Group differences were detected across rats that were treated with the 1.5 mg/kg dose of mecamylamine. Post-hoc analyses revealed that corticosterone was higher in intact females as compared to OVX (@$p \leq 0.05$), but not male rats ($p$=0.41). Group differences were detected across rats that were treated with the 3.0 mg/kg dose of mecamylamine. Post-hoc analyses revealed that corticosterone was higher in intact females as compared to both male and OVX rats (†$p \leq 0.05$).

*Figure 2* displays E2 (A) and progesterone (B) levels in intact nicotine-treated females that received mecamylamine (3.0 mg/kg) and were tested during various phases of the estrous cycle. With regard to E2, a 1-way ANOVA revealed a significant interaction between group and treatment condition [$F(3,34)=3.63$; $p \leq 0.05$]. Post-hoc analyses revealed that females tested in estrus displayed lower E2 as compared to rats that were tested in all of the other phases combined (†$p \leq 0.05$). With regard to progesterone, a 1-way ANOVA revealed a significant interaction between group and treatment condition [$F(3,34)=12.20$; $p \leq 0.05$]. Post-hoc analyses revealed that females tested in estrus displayed higher progesterone levels as compared to rats that were tested in all of the other phases combined (†$p \leq 0.05$).

*Figure 3* displays physical signs (A), EPM (B), LDT (C), and corticosterone levels (D) in intact nicotine-treated females that received mecamylamine (3.0 mg/kg) and were tested during various phases of the estrous cycle. With regard to physical signs, a 1-way ANOVA revealed that there were no main effects of estrous cycle [$F(3, 34) = 0.42$; $p=0.74$].With regard to the EPM data, a 1-way ANOVA of % time in open arms revealed a main effect of estrous cycle [$F(3, 34)=4.96$; $p\leq0.05$]. Post-hoc analyses revealed that females that were tested in estrus displayed greater % time in the open arms as compared to females (†) that were tested in proestrus, metestrus, and diestrus (†$p\leq0.05$). With regard to the LDT data, a 1-way ANOVA of % time in the lit compartment revealed a main effect of estrous cycle [$F(3, 34)=3.14$; $p\leq0.05$]. Post-hoc analyses revealed that females tested in estrus displayed greater % time in the lit compartment as compared to females in proestrus, metestrus, and diestrus (†$p\leq0.05$). With regard to corticosterone, a 1-way ANOVA revealed a main effect of estrous cycle [$F(3, 34)=3.11$; $p\leq0.05$]. Post-hoc analyses revealed that females that were tested in estrus displayed lower corticosterone as compared to all other phases combined (†$p\leq0.05$).

*Table 1* displays correlation values (Pearson's r) between behavioral measures of withdrawal or corticosterone with ovarian hormone levels (E2, progesterone, and progesterone/E2 ratios). With regard E2, there was no correlation between E2 and physical signs of withdrawal ($r=0.29$, $p=0.07$). However, there was a significant negative correlation between E2 and % time in open arms of the EPM ($r=-0.38$; $p\leq0.05$). There was a negative correlation between E2 and % time in the lit compartment of the LDT ($r=-0.32$; $p\leq0.05$). There was a positive correlation between E2 and corticosterone ($r=0.35$; $p\leq0.05$). With regard to progesterone, there was no correlation between progesterone and physical signs of withdrawal ($r=-0.16$; $p=0.35$). There was a positive correlation between progesterone and % time in open arms of the EPM ($r=0.44$; $p\leq0.05$); however, there was

no correlation between progesterone and % time in the lit compartment of the LDT (r=0.22; $p$=0.26). There was a negative correlation between progesterone and corticosterone (r=-0.43; $p \leq 0.05$). With regard to the progesterone/E2 ratios, there was a negative correlation between progesterone/E2 and physical signs of withdrawal (r=-0.36; $p \leq 0.05$). There was a positive correlation between progesterone/E2 and % time in open arms of the EPM (r=0.36; $p \leq 0.05$). However, there was no correlation between progesterone/E2 and in % time in the lit compartment of the LDT (r=0.18; $p$=0.26). Lastly, we observed a negative correlation between progesterone/E2 and corticosterone (r=-0.36; $p \leq 0.05$).

*Figure 4* reflects E2 and progesterone levels in the 2 groups of intact females that were derived from the cluster analysis. This statistical method partitions individual observations into clusters with the nearest mean value. Although this approach can result in any number of clusters, the analysis of our data revealed 2 groups of intact females that displayed high E2/low progesterone or low E2/high progesterone levels. In order to assess whether these clusters are statistically different from each other, we compared E2 and progesterone between these 2 groups. With regard to E2, an independent-samples t-test revealed a significant difference between the high E2/low progesterone versus the low E2/high progesterone group ($t(36)=3.17$, $p \leq 0.05$). With regard to progesterone, there was a significant difference between the low E2/high progesterone versus the high E2/low progesterone group ($t(36)=16.2$, $p \leq 0.05$).

*Figure 5* displays physical signs (A), EPM (B), LDT (C), and corticosterone levels (D) following administration of mecamylamine (3.0 mg/kg) in nicotine-dependent females that displayed either high E2/low progesterone or low E2/high progesterone. With regard to physical signs, there were no differences in physical signs between the high E2/low progesterone versus low E2/high progesterone groups ($t(36)=1.14$, $p$=0.26). With regard to the EPM data, females that

displayed high E2/low progesterone displayed less % time in the open arms of the EPM as compared to females that displayed low E2/high progesterone ($t(36)=3.50$; $p \leq 0.05$). With regard to the LDT data, females displaying high E2/low progesterone spent less time in the lit compartment as compared to females that displayed low E2/high progesterone ($t(36)=3.24$, $p \leq 0.05$). With regard to corticosterone, females displaying high E2/low progesterone had higher corticosterone as compared to females that displayed low E2/high progesterone ($t(36)=2.12$, $p \leq 0.05$).

**3.2 Aim 2**

*Figure 6* displays physical signs (A), EPM (B), LDT (C), and corticosterone levels (D) in nicotine-treated OVX females that were supplemented with vehicle (OVX-vehicle), E2 (OVX-E2), or both E2 and progesterone (OVX-E2+progesterone) and received mecamylamine (0 or 3.0 mg/kg) on the test day. With regard to physical signs, there were no interaction effects between group and treatment condition [$F(2,61)=1.96$; $p=0.15$]. However, there was a main effect of treatment condition [$F(1,61)=441.95$; $p \leq 0.05$], with rats that received the 3.0 mg/kg dose of mecamylamine displaying more physical signs as compared to saline controls (*$p \leq 0.05$). There were no differences in withdrawal signs across OVX-vehicle, OVX-E2, or OVX-E2+progesterone groups.

With regard to the EPM data, a 2-way ANOVA revealed a significant interaction between group and treatment condition [$F(2,61)=3.70$; $p \leq 0.05$]. Post-hoc analyses revealed that % time in open arms was lower in rats that received the 3.0 mg/kg dose of mecamylamine as compared to saline controls, an effect that was significant in OVX-vehicle, OVX-E2, and OVX-E2+progesterone rats (*$p \leq 0.05$). Group differences were detected in OVX rats that received the

3.0 mg/kg dose of mecamylamine. Post-hoc analyses revealed that % time in open arms was lower in OVX-E2 rats as compared to OVX-vehicle and OVX-E2+progesterone (†$p \leq 0.05$) rats.

With regard to the LDT data, a 2-way ANOVA revealed a significant interaction between group and treatment condition [$F(2,61)=4.82$; $p \leq 0.05$]. Post-hoc analyses revealed a significant decrease in % time in the lit compartment in rats that received the 3.0 mg/kg dose of mecamylamine as compared to saline controls, an effect that was significant in OVX-vehicle, OVX-E2, and OVX-E2+progesterone rats (*$p \leq 0.05$). Group differences were detected in rats that received the 3.0 mg/kg dose of mecamylamine. Post-hoc analyses revealed that % time in the lit compartment was lower in OVX-E2 rats as compared to OVX-vehicle and OVX-E2+progesterone rats (†$p \leq 0.05$).

With regard to corticosterone, a 2-way ANOVA revealed that there were no interaction effects between group and treatment condition [$F(2, 61)=0.91$; $p=0.12$]. However, there was a main effect of treatment condition [$F(1,61)=15.56$; $p \leq 0.05$], with rats that received the 3.0 mg/kg dose of mecamylamine displaying higher corticosterone as compared to saline controls (*$p \leq 0.05$). Also, there was a main effect of group [$F(1,61)=20.80$; $p \leq 0.05$]. Post-hoc analyses revealed that regardless of treatment condition, OVX-E2+progesterone rats displayed higher corticosterone levels than OVX-vehicle and OVX-E2 rats (#$p \leq 0.05$).

# Chapter 4: Discussion

## 4.1 Summary

The major finding is that intact female rats displayed greater indices of stress produced by nicotine withdrawal as compared to males, an effect that was ovarian hormone-dependent. Specifically, intact females displayed greater anxiety-like behavior and higher corticosterone levels during withdrawal as compared to males and OVX females. With regard to differences in nicotine withdrawal across the estrous cycle, intact females that were tested in estrus (when E2 levels are relatively low) displayed less anxiety-like behavior and corticosterone as compared to all other phases. In intact females, the magnitude of anxiety-like behavior and corticosterone levels during withdrawal were positively correlated with E2 and negatively correlated with progesterone and the progesterone/E2 ratio. In an assessment of E2 and progesterone levels in intact females, 2 clusters emerged displaying either high E2/low progesterone or low E2/high progesterone. Females displaying high E2/low progesterone levels showed greater anxiety-like behavior and corticosterone during withdrawal as compared to females displaying low E2/high progesterone. Lastly, OVX rats that received E2 supplementation displayed greater anxiety-like behavior as compared to OVX rats that received vehicle. OVX rats that received E2+ progesterone supplementation did not differ in anxiety-like behavior as compared to OVX rats that received vehicle. All groups of OVX rats displayed an increase in corticosterone levels regardless of treatment condition.

## 4.2 Is nicotine withdrawal sex- and ovarian hormone-dependent?

With regard to *sex differences* in nicotine withdrawal, intact females displayed similar levels of physical signs as compared to males, consistent with previous studies from our laboratory (Torres et al., 2013; Correa et al., 2019) and others using similar experimental conditions

(Hamilton et al., 2009). However, we acknowledge that the latter report also found that male rats display more physical signs of withdrawal in a dimly lit test room, suggesting that sex differences in the magnitude of the physical signs of withdrawal may differ depending on the lighting conditions of the test room. During nicotine withdrawal, we also observed that intact females displayed greater anxiety-like behavior and corticosterone levels as compared to males. Specifically, intact females displayed less time in the open arms of the EPM and the lit compartment of the LDT box, and higher corticosterone levels as compared to males. These findings are consistent with previous work in our laboratory showing that during nicotine withdrawal, female rats display higher levels of anxiety-like behavior (Torres et al., 2013). Reports from other laboratories have also revealed that female rats display higher serum levels of corticosterone and adrenocorticotropic hormone during nicotine withdrawal as compared to males (Gentile et al., 2011; Skwara et al., 2012). The notion that female rodents experience greater negative affective states during withdrawal is consistent with the finding that both female rats and mice display a greater aversion to an environment paired previously with nicotine withdrawal (Kota et al., 2007, 2008; O'Dell and Torres, 2014).

With regard to the role of *ovarian hormones* in nicotine withdrawal, OVX females displayed similar physical signs as compared to intact females. Interestingly, OVX females displayed less anxiety-like behavior than intact females, consistent with previous work in our laboratory (Torres et al., 2015). This suggests that ovarian hormones play an important role in modulating strong stress responses (but not physical signs) produced by nicotine withdrawal in female rats. The different pattern of results in OVX rats suggests that physical signs and negative affective states produced by withdrawal are modulated via distinct brain mechanisms, a suggestion that has been put forth in recent review papers (Malin et al., 2017; Molas et al., 2017).

### 4.3 Is the magnitude of nicotine withdrawal estrous cycle-dependent?

In the present study, the physical signs of withdrawal were similar across the 4 phases of the estrous cycle, a finding that is not consistent with a recent report showing that physical signs are higher in metestrus versus proestrus female rats (Henceroth et al., 2018). The present study also found that estrus females displayed less anxiety-like behavior and corticosterone levels as compared to all other phases. Specifically, estrus females spent more time in the open arms of the EPM and the lit compartment of the LDT box and displayed lower corticosterone levels as compared to all other phases. Following chronic social stress, one report revealed that estrus female rats display less anxiety-like behavior in the EPM as compared to diestrus females (McCormick et al., 2008). Shansky et al., (2004) also found that estrus females displayed less impairments on a learning task following a pharmacological stressor as compared to proestrus females. Also, other studies found that corticosterone levels were lower in estrus females following presentation of an acute stressor as compared to proestrus females (Viau and Meaney 1991; Conrad et al., 2004). Baseline levels of anxiety-like behavior have also been shown to be lower in estrus versus diestrus females (Frye & Rhodes et al., 2006; Gouveia et al., 2004; Marcondes et al., 2001; Mora et al., 1996). Also, corticosterone release is lower in estrus versus proestrus females following acute restraint stress (Figueiredo et al., 2002). Together, these studies suggest that stress responses may be lower during the estrus phase, perhaps because this is a fertile phase of the cycle that is also characterized by greater exploratory behaviors that promote mating behavior (Frye and Rhodes et al.,2006; McCormick et al., 2008). The present study also revealed that estrus females displayed lower E2 and higher progesterone levels as compared to all other phases. These findings suggest that the magnitude of nicotine withdrawal may be greater during phases of the cycle when E2 is lower and progesterone is higher relative to the other phases of the estrous cycle.

### 4.4 Is the magnitude of nicotine withdrawal related to E2 and progesterone levels?

The present study correlated each intact female rat's hormone levels with 4 different outcomes (physical signs, EPM, LDT, and corticosterone). E2 and progesterone levels did not appear to be correlated with physical signs produced by withdrawal. Anxiety-like behavior and corticosterone levels were positivity correlated with E2 and negatively correlated with progesterone levels. This effect was not observed in our measures of anxiety-like behavior on the LDT, an effect that may be related to fluctuating hormone levels across individual rats. Indeed, the cluster analysis allowed us to compare our measures of withdrawal in groups of animals that were categorized by high E2/low progesterone or low E2/high progesterone. Indeed, our analysis of these groups revealed that rats displaying high E2/low progesterone displayed greater anxiety-like behavior in both the EPM and LDT tests. These findings suggest that E2 promotes and progesterone reduces the magnitude of anxiety-like behavior and corticosterone levels during nicotine withdrawal. To our knowledge, the present study is the first assessment of the relationship between ovarian hormone levels and the magnitude of nicotine withdrawal in rodents.

### 4.5 What are the effects of hormone supplementation on nicotine withdrawal in OVX rats?

In the present study, the physical signs of withdrawal were similar in OVX rats that received vehicle, E2, or E2+progesterone supplementation. These data further support our assertion that ovarian hormones do not play a role in physical signs produced by nicotine withdrawal. Interestingly, the data also revealed that OVX rats that received E2 supplementation displayed higher levels of anxiety-like behavior as compared to OVX rats that received vehicle. Specifically, during nicotine withdrawal, OVX-E2 rats spent less time in the open arms of the EPM and the lit compartment of the LDT box as compared to OVX-vehicle rats. This finding is consistent with our assertion from Aim 1 that E2 promotes anxiety-like behavior produced by

nicotine withdrawal. The cytology of the OVX-E2 rats revealed that these animals were tested in a proestrus-like phase. This is of interest given that high levels of anxiety-like behavior were observed in the proestrus rats of Aim 1 and the OVX-E2 animals of Aim 2. The results revealed that OVX-E2+progesterone rats displayed less anxiety-like behavior as compared to the OVX-E2 rats. This might have been related to the direct anxiolytic effects of progesterone given that this hormone was administered 4.5 hour prior to the test. Also, the cytology of the OVX-E2+progesterone rats revealed that these animals were tested in an estrus-like phase. The lower levels of anxiety-like behavior in this group is consistent with the estrus females in Aim 1. Thus, our ovarian supplementation procedures in Aim 2 induced cytology patterns and behavioral outcomes that mimicked the pattern of results in intact females that were tested in estrus and proestrus in Aim 1.

**4.6 Major conclusions and significance**

The major conclusion of this dissertation is that E2 increases anxiety-like behavior during nicotine withdrawal in females. This assertion is based on our finding that anxiety-like behavior was lowest in the intact females that were tested in the estrus phase, when E2 levels are the lowest (Bohler., et al., 1990; Nappi et al., 1997). In our assessment of ovarian hormone levels, we also found that estrus females displayed lower levels of E2 as compared to rats tested in all other phases of the estrous cycle. Also, in intact females, E2 levels were positively correlated with anxiety-like behavior during nicotine withdrawal. Lastly, OVX rats that received E2 supplementation also displayed higher anxiety-like behavior as compared to OVX rats that received vehicle. This finding is consistent with a previous report showing that OVX rats that received E2 supplementation displayed greater sensitivity to pharmacological stressor as compared to OVX rats that received vehicle (Shansky et al., 2004). The ability of E2 to increase sensitivity to a stressful stimulus in

females may be due to the direct effects of E2 on the hypothalamus pituitary adrenal (HPA) axis. This is based on our finding that OVX rats displayed reduced basal corticosterone levels and this is reversed following E2 replacement. Also, previous work revealed that the increase in corticosterone levels produced by acute stress are greater in OVX rats that received E2 supplementation (Handa & Weiser, 2014; Green et al., 2018). Also, the highest levels of the stress hormone, corticotropin releasing factor (CRF) were observed during proestrus, when E2 levels are highest (Bohler et al., 1990; Nappi et al., 1997). The present finding suggests that E2 promotes anxiety-like behavior produced by nicotine withdrawal in females.

Another conclusion of the present study is that progesterone appears to decrease anxiety-like behavior produced by nicotine withdrawal in females. This assertion is based on our finding that intact female rats displaying high levels of progesterone display less anxiety-like behavior and corticosterone release relative to intact females displaying lower levels of progesterone. Also, our OVX rats that received E2+progesterone displayed less anxiety-like behavior as compared to OVX rats that received E2 alone, suggesting that progesterone may have reduced the intense anxiety observed in the OVX+E2 rats during withdrawal. Previous work has shown that progesterone administration decreases anxiety-like behavior in intact and OVX mice (Mora et al., 1996; Reddy et al., 2005). These data suggest that progesterone decreases anxiety-like behavior produced by nicotine withdrawal in females.

The work in this dissertation is significant because it reflects an important first step towards understanding the role of ovarian hormones modulate tobacco use in females. This dissertation revealed that E2 promotes anxiety-like behavior, whereas progesterone reduces stress produced by nicotine withdrawal. The results of this study might inform the development of more effective tobacco cessation strategies in females. For example, one might expect a medical professional to

assess the hormone status of a women who is contemplating quitting smoking. Our data suggest that the best time to quit smoking may be in phases of the cycle when E2 levels are lowest and progesterone levels are relatively higher in order to minimize the extent to which E2 may intensify the nicotine withdrawal syndrome. Indeed, a clinical report revealed that women have an easier time quitting smoking during the luteal phase, when E2 levels are decreasing and progesterone levels are relatively higher (Allen et al., 2008). Future studies are need to more fully understand the underlying biological factors that modulate the aversive effects nicotine withdrawal and promote tobacco use in females. This work will be an essential step towards developing more effective cessation strategies that will reduce health disparities produced by tobacco use in women.

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**Table 1.** Correlation values (Pearson's r) between behavioral measures of nicotine withdrawal or corticosterone levels with ovarian hormone levels

| Measure | E2 | Progesterone | Progesterone/E2 ratio |
|---|---|---|---|
| Physical signs | 0.30 | -0.16 | -0.36* |
| Anxiety – like behavior | | | |
| EPM | -0.40* | 0.44* | 0.35* |
| LDT | -0.32 | 0.23 | 0.18 |
| Corticosterone | 0.35* | -0.44* | -0.37* |

* Reflects a significant correlation ($p \leq 0.05$).

**Table 1.** Correlations between measures of nicotine withdrawal and E2, progesterone, or progesterone/E2 ratios in intact females that received the 3.0 mg/kg dose of mecamylamine. There was a negative correlation between physical signs and the progesterone/E2 ratio. There was a negative correlation between anxiety-like behavior on the EPM and E2. There was a positive correlation between corticosterone and E2. There was a positive correlation between anxiety-like behavior on the EPM and progesterone and the progesterone/E2 ratio. There was a negative correlation between corticosterone and progesterone and the progesterone/E2 ratio.

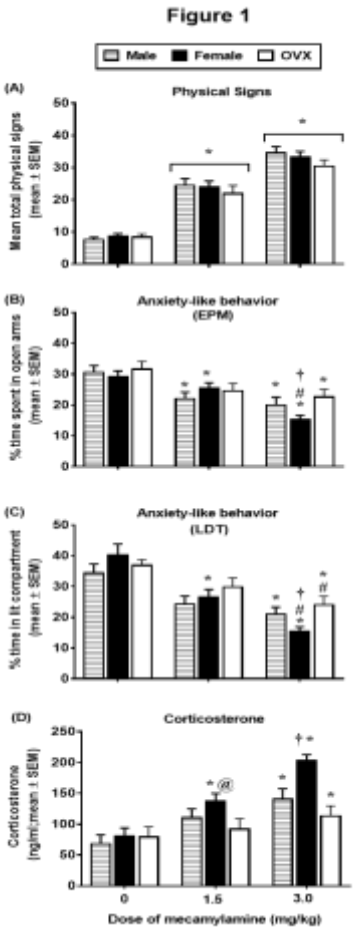


**Figure 1.** Physical signs (A), % time in the open arms of the EPM (B), % time in the lit compartment of the LDT box (C) and corticosterone (D) (±SEM) in nicotine-treated male (0 mg/kg n=16; 1.5 mg/kg n=17; 3.0 mg/kg n=12), intact female (0 mg/kg n=18; 1.5 mg/kg n=24; 3.0 mg/kg n=38), and OVX (0 mg/kg n=13; 1.5 mg/kg n=12; 3.0 mg/kg n=13) rats that received saline or mecamylamine (1.5 or 3.0 mg/kg). Asterisks (*) denote a difference from respective saline controls, number signs (#) denote a difference from their respective mecamylamine-treated group, at signs (@) denote a difference from OVX rats in their respective treatment condition, and the dagger (†) denotes a difference from males and OVX rats in their respective treatment condition ($p \leq 0.05$).

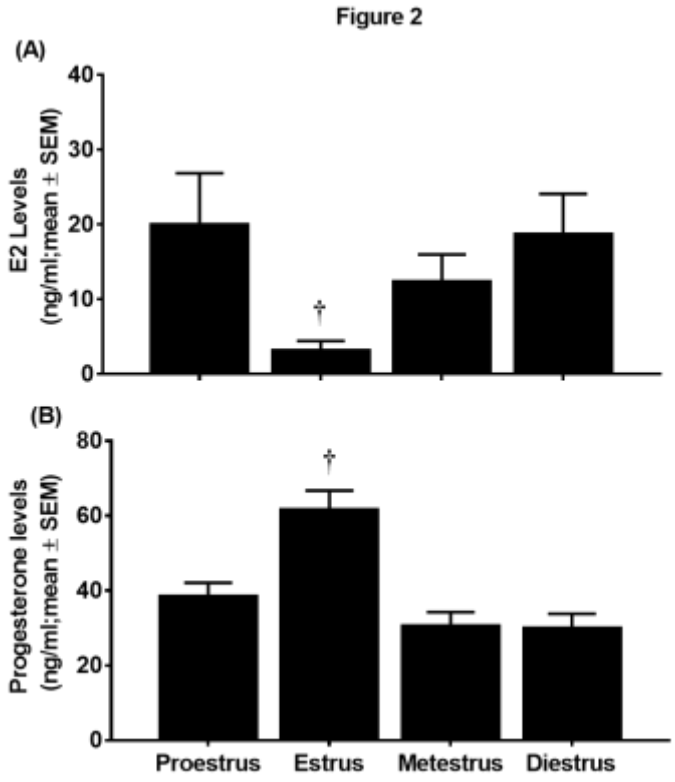


**Figure 2.** E2 (A) and progesterone (B) levels (±SEM) during withdrawal produced by mecamylamine (3.0 mg/kg) in intact females rats that were tested during the proestrus (n=12), estrus (n=12), metestrus (n=7), and diestrus (n=7) phase of the estrous cycle. Daggers (†) denotes a significant difference from all other groups ($p \leq 0.05$).

Figure 3

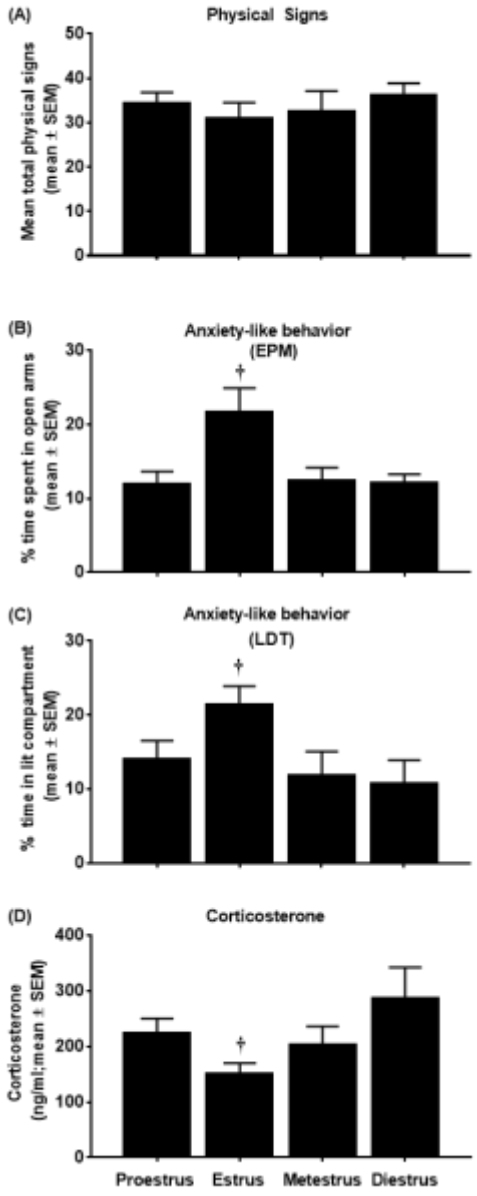


**Figure 3.** Physical signs (A), % time in the open arms of the EPM (B), % time in the lit compartment of the LDT box (C) and corticosterone (D) (±SEM) during withdrawal produced by mecamylamine (3.0 mg/kg) in intact females rats that were tested during the proestrus (n=12), estrus (n=12), metestrus (n=7), and diestrus (n=7) phase of the estrous cycle. Daggers (†) denotes a difference from all other groups ($p \leq 0.05$).

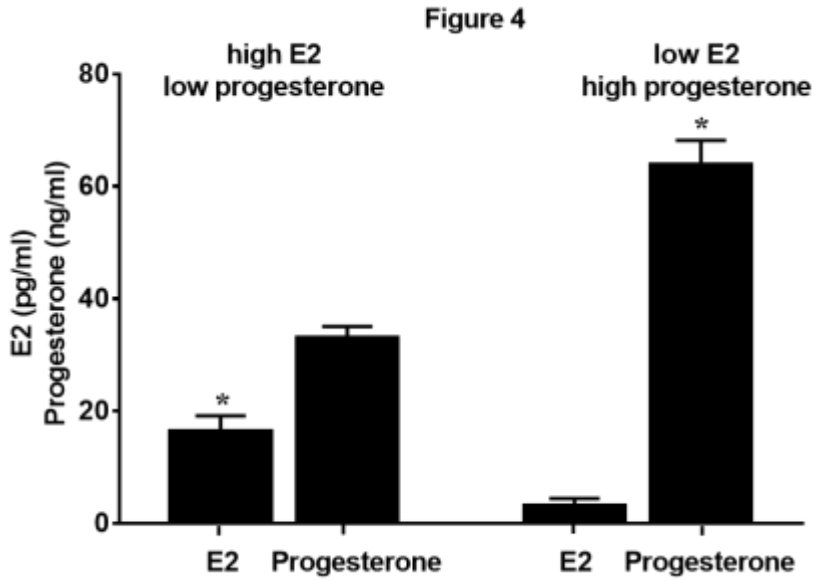


**Figure 4.** E2, and progesterone levels (±SEM) in female rats that displayed high E2/low progesterone (n=26) or low E2/high progesterone (n=12) that received mecamylamine (3.0 mg/kg) on test day. Asterisk (*) denotes a significant difference from the other hormone group ($p \leq 0.05$).

Figure 5

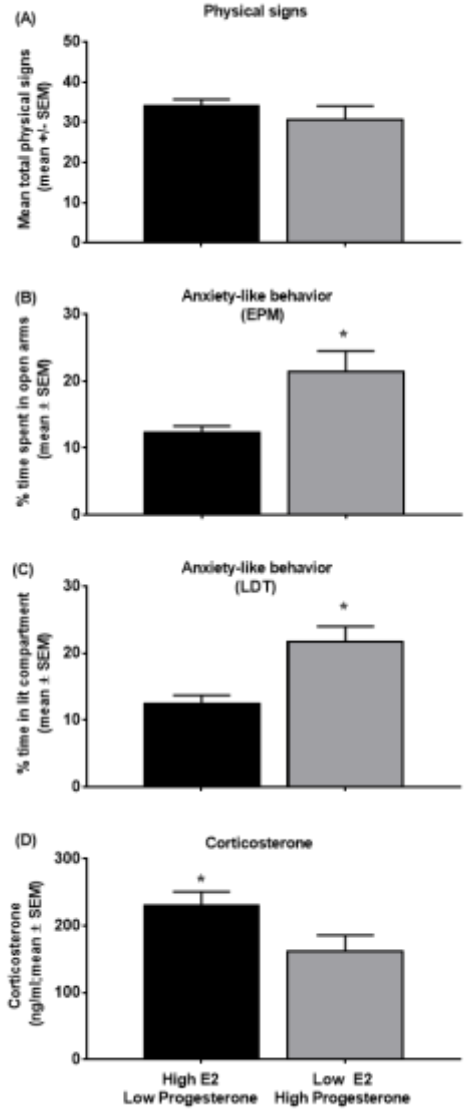


**Figure 5.** Physical signs (A), % time in the open arms of the EPM (B), % time in the lit compartment of the LDT box (C) and corticosterone (D) (±SEM) in female rats displaying high E2/low progesterone (n=26) or low E2/high progesterone (n=12) that received mecamylamine (3.0 mg/kg) on test day. Asterisks (*) denote a difference between groups ($p \leq 0.05$).

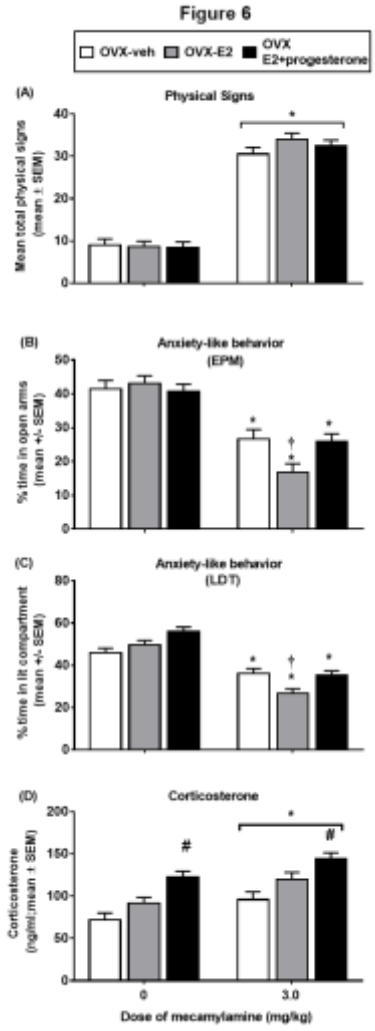


**Figure 6.** Physical signs (A), % time in the open arms of the EPM (B), % time in the lit compartment of the LDT box (C) and corticosterone (D) (±SEM) in nicotine-treated OVX rats that received vehicle (OVX-veh; 0 mg/kg n=10; 3.0 mg/kg n=8), E2 (OVX-E2; 0 mg/kg n=13 mg/kg; 3.0 mg/kg n=10) or E2+progesterone (OVX-E2+progesterone; 0 mg/kg n=13; 3.0 mg/kg n=13) that received mecamylamine (0 or 3.0 mg/kg) on the test day. Asterisks (*) denote a difference from saline controls, number signs (#) denote a difference from OVX-veh and OVX-E2 regardless of mecamylamine treatment, and the dagger (†) denotes a difference from OVX-veh and OVX-E+progesterone rats in their respective treatment condition ($p \leq 0.05$).

## Curriculum Vitae

Rodolfo J. Flores Garcia was born to Rodolfo Flores and Martha Garcia in Naucalpan, Estado de México, México. He graduated from San Pedro High School in San Pedro, California in May 2008 and entered California State University, Long Beach (CSULB) in Long Beach, California, the following semester. During his undergraduate career, Rodolfo worked as a tutor in the Boys and Girls Club of America where he mentored students from diverse backgrounds. He became interested in Neuroscience and began research work with Dr. Arturo R. Zavala who studied the long-term effects of early age exposure to methylphenidate in rats. He became interested in investigating the factors that make individuals more vulnerable to become addicted to drugs. Therefore, in the summer of 2013, he joined the laboratory of Dr. Laura E. O'Dell at the University of Texas at El Paso (UTEP) as part of a summer research internship. Her laboratory combines behavioral, molecular and biochemical techniques to study the factors that promote tobacco use in vulnerable populations. He obtained his Bachelor of Arts degree in Psychology with a minor in Chemistry from CSULB in May 2014. Thereafter, he entered the Social, Cognition, and Neuroscience program at UTEP in August 2014, where he received mentorship from Dr. Laura E. O'Dell to investigate the factors that promote tobacco use in females. Rodolfo received his Master's degree in Experimental Psychology during the Spring semester of 2017. He then continued his training and research with Drs. O'Dell and Ian Mendez in the Social, Cognition, and Neuroscience doctoral program at UTEP. Rodolfo has published 3 first-author articles and is co-author on 3 publications examining nicotine reward and withdrawal in animal models of addiction. He presented his research work in 25 conferences focused on drug abuse.

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