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Characterization Of The Behavioral, Biochemical And Molecular Indices Of Stress Produced By Nicotine Exposure And Withdrawal In Male And Female Rats

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CHARACTERIZATION OF THE BEHAVIORAL, BIOCHEMICAL AND MOLECULAR INDICES OF STRESS PRODUCED BY NICOTINE EXPOSURE AND WITHDRAWAL IN MALE AND FEMALE RATS

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Lovingly dedicated to my family, Luis Alfredo Torres, Maria Alma Torres, Nancy Torres and Evelyn Torres, my beautiful wife.

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by

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Abstract

Introduction: Tobacco use is a major economic and health problem. Particularly concerning is that women consume more tobacco products, have a more difficult time quitting, and are less likely to benefit from cessation therapies than men. As a result, women are at higher risk of developing tobacco-related diseases. Women are generally more susceptible to stress and are more likely to cope with stress by smoking as compared to men. During abstinence, women also experience intense anxiety as compared to men and report that the anxiety-reducing effects of smoking are the main reason for continued use and relapse. Thus, stress produced by nicotine withdrawal may enhance susceptibility to tobacco use and relapse in female versus male smokers. Although cessation approaches focus on alleviating withdrawal, the contribution of anxiety produced by withdrawal to tobacco use in females is unclear. **Methods**: The present study compared sex differences in various behavioral and biological indices of stress during nicotine exposure and withdrawal from this drug. Potential sex differences in these measures were also compared during adolescence. Briefly, male and female adolescent and adult rats underwent sham surgery or received subcutaneous implantation of pumps that delivered nicotine. Fourteen days later, the pumps were either removed to induce withdrawal or were left in place in order to examine the effects of nicotine exposure. Twenty-four hours later, anxiety-like behavior was assessed using elevated plus maze and open field tests. Blood samples were also collected and analyzed for corticosterone, a biological marker of stress. Brain tissue from the nucleus accumbens (NAcc), amygdala, and hypothalamus were examined for changes in corticotropin-releasing hormone (CRH) gene expression. Potential group differences in nicotine metabolism were assessed via measurements of cotinine (a nicotine metabolite) during nicotine exposure and withdrawal. **Results:** During withdrawal, adult females displayed higher levels of anxiety-like

behavior, plasma corticosterone, and CRH gene expression in the NAcc relative to adult males. During nicotine exposure; however, adult males exhibited higher levels of corticosterone and CRH gene expression in the amygdala. These sex differences are not related to nicotine metabolism, since male and female adult rats displayed similar cotinine levels during nicotine exposure and withdrawal. Adolescent males displayed an increase in anxiety-like behavior and an up-regulation of CRH mRNA expression in the amygdala during exposure and withdrawal from nicotine. These findings are likely related to stress produced by the high doses of nicotine that were administered to the adolescents to produce equivalent levels of cotinine as adults. **Conclusion:** The results show that there are sex differences in stress responses produced by nicotine exposure versus withdrawal from this drug. Of particular clinical relevance, these findings show that female adults display greater behavioral and biological indices of stress during nicotine withdrawal than males. Thus one plausible underlying substrate by which adult females are more vulnerable to tobacco use may be intense stress produced by nicotine withdrawal as compared to males. This work reflects an important first step toward developing effective smoking cessation strategies for treating tobacco use among women. Importantly, the pattern of changes during nicotine exposure and withdrawal were different in adult versus adolescent rats, suggesting important developmental differences that should be considered when developing smoking cessation treatments for different age groups.

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Chapter 1: Introduction

1.1 Tobacco use is a major societal problem

Tobacco use is a major health and economic concern with immense negative impacts on our society. Tobacco use is reported to be the number one cause of preventable deaths in the United States, as it claims the lives of over 400,000 individuals each year (Centers for Disease Control and Prevention [CDC], 2008; 2011). Long-term tobacco use leads to deleterious health consequences such as lung cancer, emphysema, and a variety of cardiovascular diseases (D'Alessandro et al., 2012; Hecht, 2012; Milara & Cortijo, 2012). In addition, the consumption of tobacco products is reported to have a health-care cost of over $97 billion dollars per year (CDC, 2010b). Given the magnitude of the problem, much research is needed to understand the various factors that contribute to tobacco use.

Women are more susceptible to tobacco use than men: Several clinical reports have suggested that women are more susceptible to tobacco use as compared to men (Pauly, 2008; Perkins & Scott, 2008; Perkins et al., 2012; Schnoll et al., 2007). For example, women smokers are reported to consume more tobacco products relative to men (Cropsey et al., 2008; Hammond, 2009). Women also exhibit lower rates of quitting and are less likely to benefit from nicotine replacement therapies (NRTs) than men (Cepeda-Benito et al., 2004; Perkins et al., 2001; 2008; Perkins & Scott, 2008; Piper et al., 2010; Schnoll et al., 2007). During abstinence from smoking, women report greater levels of anxiety, depression, and stress (Perkins & Scott, 2008; Schnoll et al., 2007; Xu et al., 2008) and higher levels of cortisol (a biological marker of stress in humans) as compared to men (Hogle & Curtin, 2006). Clinical studies have also shown that women report more often that the anxiety-reducing effects of cigarettes are the main reason for continued smoking and relapse as compared to men (Perkins & Scott, 2008; Perkins et al., 2009; 2012;

Piper et al., 2010). Unfortunately, women smokers are also at a higher risk of developing tobacco-related diseases, including various cancers and cardiovascular disorders (Kiyohara & Ohno, 2010; Langhammer et al., 2000; 2003). Collectively, these studies suggest that women are more susceptible to tobacco use than men. Despite the well-recognized problem of smoking in women, there is still a critical knowledge gap regarding the factors that contribute to enhanced vulnerability to tobacco use in women.

Adolescents are more susceptible to tobacco use than adults: Epidemiological studies have also shown that adolescents are more susceptible to tobacco use than adults (Carpenter et al., 2010; Chung et al., 2011; Moolchan et al., 2005). In the past ten years, smoking rates have declined in the general population; however, there has been a steady rise in the rate of smoking among adolescents (National Institute on Drug Abuse [NIDA], 2011). There are about one million new adolescent smokers each year, and it is estimated that about 20 percent of high school seniors are daily smokers (Faraday et al., 2003; James-Walke et al., 2007). It is estimated that 90 percent of smokers initiated smoking behavior before the age of 18 (United States Department of Health and Human Services [USDHHS], 2004). Studies on the motivational factors contributing to smoking in adolescents point to a number of influencing factors including affective disorders, cultural tolerance, peer pressure, and anxiety management (Baker et al., 2004a; Corrigall et al., 2001; Hurt et al., 2000; Jaszyna-Gasior et al., 2009; McGue et al., 2000). Reports have also shown that NRTs are *less* effective in reducing smoking rates among adolescents versus adults (Cepeda-Benito et al., 2004; Hurt et al., 2000; Myers et al., 2006; Rosen & Maurer, 2008; Stanton, 1995). There is also evidence showing that adolescent tobacco exposure increases the likelihood of tobacco use during adulthood (Baker et al., 2004b; DiFranza & Wellman, 2003; Lessov-Schlaggar et al., 2008; Maharaj & Ternullo, 2001). As a consequence

of long-term tobacco use, smokers who initiate smoking during adolescence are more likely to suffer from negative health consequences, such as lung cancer and other pulmonary diseases (Caldeira et al., 2012; Maritz & Mutemwa, 2012).

During adolescence, females are more susceptible to tobacco use than males: A closer review of the literature reveals that adolescent females are even more vulnerable to tobacco use as compared to adolescent males. Nationwide, 15.2 percent of adolescent females in the ninth grade use cigarettes every day in comparison to 12 percent of adolescent males (CDC, 2010a; American Lung Association, 2004). Adolescent females are also more likely to smoke cigarettes and exhibit lower quit rates as compared to adolescent males (Anderson & Burns, 2000; James-Walke, et al. 2007; Perkins, 2001). Adolescent females also report higher levels of stress and depression following nicotine cessation as compared to their male counterparts (Colby et al., 2000; Nichter et al., 1997). As a consequence of early tobacco use, adolescent females are at an even greater risk of developing diseases associated with chronic smoking during adulthood such as lung and breast cancer (USDHHS, 2004). Despite the fact that female adolescents are more susceptible to tobacco use, few studies have focused on examining nicotine withdrawal in this population.

Based on these findings, there is evidence that women, during different stages of development, are more susceptible to tobacco use relative to their male counterparts. Few studies have focused on understanding how various factors, such as anxiety and stress contribute to enhanced tobacco use in females. This critical knowledge gap presents an obstacle for developing effective treatment strategies for smoking cessation in adult and adolescent female smokers. Understanding how stress and anxiety contribute to tobacco use is an *important first step* toward developing more effective treatment strategies for female smokers. Thus, this

dissertation compares behavioral indices of anxiety and biological markers of stress in adult and adolescent male and female rats during nicotine exposure and withdrawal from this drug.

1.2 Definition of stress and anxiety

Although the terms stress and anxiety are often used interchangeably, they have different definitions. The term stress has been defined as a physical and psychological response elicited by a changing environment in which the body is forced to adapt to new conditions (see Selye, 1975a; 1975b). A stress response is the result of a perceived or actual threatening situation that leads to adverse physiological and psychological consequences (Sarnyai et al., 2001; Ulrich-Lai & Herman, 2009). One definition suggests that a state of stress is induced as the result of a complex appraisal process whereby the demands of a novel situation exceed the body's available resources (Dedovic et al., 2009). In short, stress is a response elicited by environmental challenges that endanger or are perceived to threaten preservation of homeostasis (Szabo et al., 2012). For the purposes of this dissertation, the term stress is limited to biological responses produced by environmental demands that produce homeostatic changes (Selye, 1977), which can occur in rodents with behavioral manifestations that are believed to model anxiety-like behavior in humans.

The term anxiety is often used to describe a negative mood state that is induced by an ongoing or anticipated aversive event (Craske et al., 2009; Ulrich-Lai & Herman, 2009). The term anxiety is also closely associated with the emotions of worry and fear in preparation for negative consequences (Barlow, 2002; Zvolensky & Schmidt, 2007). The long-term consequences of chronic anxiety are panic attacks, emotional disruption, and even memory loss (Bourke et al., 2012). A report from the American Psychological Association [APA] (2009) suggests that between 40 to 50 percent of individuals experiencing high levels of anxiety suffer

from negative health effects such as sleep loss, irritability, fatigue, headaches, and depression. For the purposes of this dissertation, the term anxiety is reserved to describe a negative psychological mood state that occurs in humans (Barlow et al., 1986; Barlow, 2002).

A report from the APA (2012) found that 75 percent of adults in the United States experience high levels of anxiety at least once every month. Stress is also common in adolescents, as 45 percent of adolescents also report experiencing intense stress at least once a month in the United States (APA, 2009). Despite the prevalence and negative health consequences of anxiety, our society has been unable to develop proper skills to reduce anxiety.

1.3 Tobacco use and anxiety

Smoking is used to cope with anxiety: Much work has shown that smoking is a common tool used to cope with anxiety (Aronson et al., 2008; Park & Breland, 2007; Parrott & Murphy, 2012; Perkins at al., 2010; Slopen et al., 2012). Self-report studies have indicated that the main reason why people use tobacco is to reduce anxiety and induce a state of relaxation (Aronson et al., 2008, Fidler & West, 2009; McEwen et al., 2008; Parrott et al., 2012; Perkins at al., 2010). Furthermore, smokers report they do not quit smoking because cigarettes help them to cope with the high levels of anxiety in their lives (Perkins et al., 2012; 2010; Dupont et al., 2012). A nation-wide survey also indicates that people primarily use cigarettes to manage their anxiety levels (APA, 2012). College students also report smoking cigarettes to alleviate anxiety and frustration, but not to induce positive mood states such as happiness or satisfaction (Brown et al., 2011). Even children report that their smoking attempts were related to coping with anxiety (Hruba & Zaloudikova, 2010). A recent review also concluded that anxiety is a major contributing factor that leads to enhanced vulnerability to long-term tobacco use (for a review, see Bruijnzeel, 2012). Additionally, it has been shown that smokers display higher basal levels of

cortisol as compared to non-smokers (Mendelson et al., 2005; 2008; Steptoe & Ussher, 2006). Although there are many factors that contribute to smoking behavior such as peer pressure, hunger suppression, and co-morbid drug use (Brown et al., 2011), the majority of evidence suggests that tobacco is used mainly to cope with anxiety (Aronson et al., 2008; Park & Breland, 2007; Parrott et al., 2012; Perkins at al., 2010; Slopen et al., 2012).

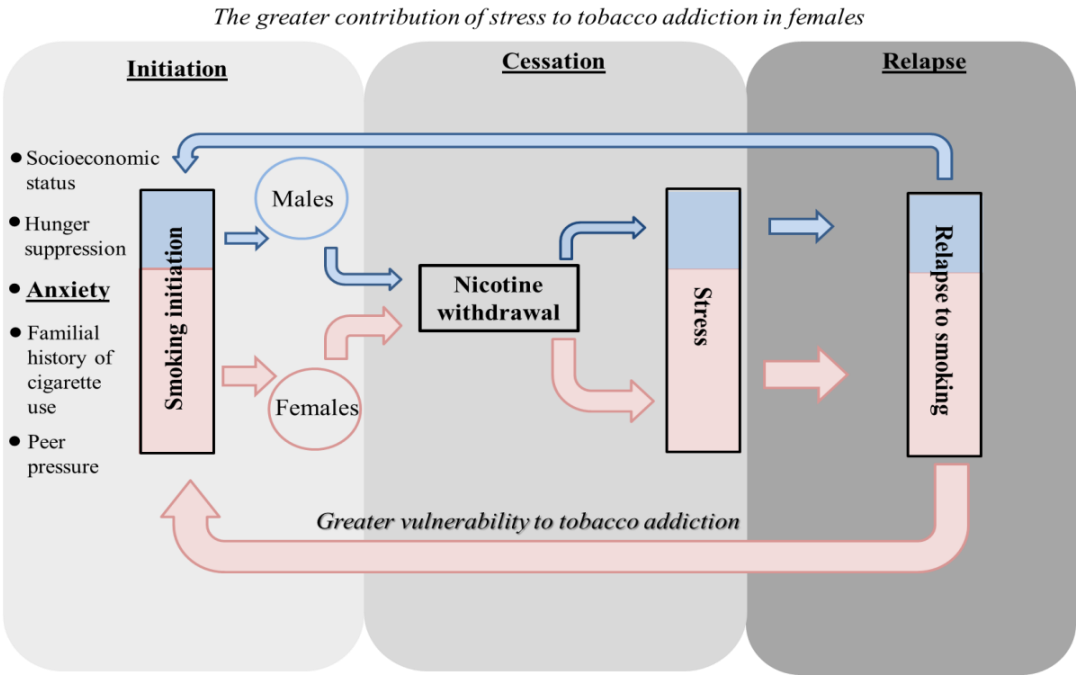
Abstinence from chronic smoking produces anxiety and stress responses: Although cigarettes are commonly used to induce a state of relaxation, long-term tobacco use produces dependence that is driven in large part by avoiding anxiety produced by withdrawal (Aronson et al., 2008; Hughes & Callas, 2010; Parrott & Murphy, 2012; Perkins et al., 2009; 2010). The signs of tobacco dependence in humans include, but are not limited to, depression, irritability, and negative affective states such anxiety (Parrott & Zeichner, 2001; Parrott et al., 2012; Pauly, 2008). The physical signs of dependence include nausea, headache, sleep disturbances and hunger (Perkins et al., 2009; 2012). A physiological stress response is also elicited during smoking abstinence. This includes an increase in blood levels of cortisol (Hogle & Curtin, 2006; Steptoe & Ussher, 2006). Studies designed to assess the motivation for smoking relapse have identified the avoidance of anxiety produced by withdrawal as the primary reason for relapse (Battista et al., 2008; Fidler & West, 2009; Lawrence et al., 2011). Consistent with this, a nationwide survey found that relapse to smoking behavior is mainly driven by high levels of anxiety produced by smoking abstinence (Aronson et al., 2008). In summary, these studies suggest that withdrawal from chronic tobacco use leads to anxiety and stress responses that contribute to smoking relapse.

Nicotine is the addictive ingredient in tobacco products: Research has shown that nicotine is the major habit forming compound in tobacco products (USDHHS, 2010). In humans,

nicotine induces a feeling of euphoria, relaxation, alertness, and motor activation (Heishman et al., 2010; Hukkanen et al., 2005; Le Foll & Goldberg, 2006; 2009; NIDA, 2009). The reinforcing effects of nicotine are well established, as nicotine is readily self-administered by humans, primates, dogs, and rodents (Caille, 2012; Corrigall, 1999; Harvey et al., 2004; Katner et al., 2004; Le Foll et al., 2007; Le Foll & Goldberg, 2006; 2009; Risner et al., 1983). Nicotine is a natural pyridine alkaloid found in tobacco leaves and is most commonly ingested through the respiratory tract (Pogocki et al., 2007). When nicotine is consumed via smoke, the acidic pH of this drug allows it to be readily absorbed into the blood stream and central nervous system (Pogocki et al., 2007). The amount of nicotine found in a given cigarette depends on many factors, including the brand of cigarette, the strain of tobacco leaf, and tobacco plant cultivation. Despite this, the range of nicotine is about 1-2 mg per cigarette (Hukkanen et al., 2005; NIDA, 2009; Sarkar et al., 2012; St. Charles et al., 2011). It is important to note that nicotine is only one of various compounds found in cigarettes, and diseases associated with tobacco use have mostly been linked to carcinogenic compounds other than nicotine (Baker et al., 2004a; 2004b). Although nicotine produces pleasurable effects, the reinforcing effects of nicotine are short lasting and relatively mild (Perkins et al., 2009; Pogocki et al., 2007). Thus, it is unlikely that acute reinforcing effects of nicotine are the only motivational factor for continued tobacco use and relapse behavior. Following chronic use however, nicotine withdrawal produces negative affective states that appear to be a main motivational factor for tobacco use and relapse (Pauly, 2008; Perkins et al., 2009; 2012). Given that the dependence-inducing effects of tobacco are closely associated with withdrawal from chronic exposure to nicotine, this dissertation focuses on animal models involving chronic administration of nicotine and withdrawal from this drug.

1.4 Proposed model of enhanced vulnerability to tobacco use in females

Proposed model: Clinical evidence suggests that women are more susceptible to tobacco use and may experience stronger withdrawal than men. Furthermore, anxiety produced by nicotine withdrawal is a strong motivational factor that contributes to smoking relapse (Pauly, 2008; Perkins et al., 2009; 2010; 2012). Thus, it is postulated that anxiety may contribute to initiation of tobacco use in females, and that greater stress produced by withdrawal leads to higher vulnerability to relapse in females.



The diagram above depicts the proposed model of causation for enhanced vulnerability to tobacco use in women. This model is derived from clinical evidence suggesting that females have a predisposition to tobacco use due to a hypersensitive response to stress during nicotine withdrawal. The color coding reflects a larger relapse effect in women (pink) versus men (blue). First, women may be more susceptible to initiate smoking behavior, because smoking is used to cope with stronger anxiety. Second, anxiety and stress produced by nicotine withdrawal are greater in women as compared to men. Because women experience greater stress during

withdrawal, more women relapse as compared to men. Lastly, greater motivation to smoking relapse leads to greater tobacco use in women. Although there are several phases of tobacco addiction in which there are sex differences, this dissertation focuses on withdrawal.

1.5 Females have a predisposition to tobacco use due to anxiety and heredity factors

Innate sex differences to anxiety: Evidence suggests that there are innate sex differences to anxiety. For example, clinical studies have shown that females are three times more likely to suffer from social anxiety then men (Bourke et al., 2012, Xu et al., 2008; Xu et al., 2012). Women also display higher rates of depression and generalized anxiety than men (Hankin & Abramson, 2001; McLaughlin et al., 2011; Pigott, 2003; Somers et al., 2006; Vesga-Lopez et al., 2008). Additionally, women have a greater susceptibility to developing anxiety disorders following a traumatic life event as compared to men (Kobayashi & Mellan, 2012; Holbrook et al., 2002; Seedat & Stein, 2000). Women also report taking more days off from work due to anxiety as compared to men (CDC, 2004). However, there are some indices of anxiety that are stronger in men than women. For example, men experience higher immune and metabolic problems during high anxiety as compared to women (Kudielka & Kirschbaum, 2005; Penninx et al., 2003; Vogelzangs et al., 2012).

Clinical reports have also suggested that adolescent females experience more anxiety following stressful life events as compared to males (CDC, 2010a; Moksnes et al., 2010). Additionally, high levels of anxiety in adolescent females have been shown to increase the risk for depression and anxiety later during adulthood, and this relationship was not seen in adolescent males (Brouke et al., 2012; Merikangas & Pine, 2002; Piccinelli & Wilkinson, 2000). Importantly, studies have found that adolescent females who experience stress are more likely to initiate drug abuse as compared to females that do not experience stress during adolescents (Jun

et al., 2010; Timko et al., 2008). In summary, these studies show that there are sex differences to anxiety, with females being more susceptible than males.

Anxiety disorders and smoking: There is evidence to suggest strong comorbidity between anxiety disorders and smoking behavior (Brujnzeel, 2005; Cougle et al., 2010; Lopes et al., 2002; Mykletun et al., 2008; Trosclair & Dube, 2010). Although people with nicotine dependence and anxiety disorders account for less than eight percent of the population, they consume over 30 percent of all cigarettes smoked in the United States (Grant et al., 2004). A survey conducted by Lasser et al. (2000) suggests that 55 percent of people meeting the criteria for an anxiety disorder are also regular smokers. These findings are consistent with other reports showing that the rate of anxiety disorders is higher in individuals who smoke as compared to non-smokers (Lawrance et al., 2010; 2011; McClave et al., 2009; Morissette et al., 2005; Mykletun et al., 2008; Sonntag et al., 2000).

People with anxiety disorders also display more symptoms of nicotine withdrawal during smoking abstinence. Watson et al. (2012) demonstrated that smokers diagnosed with a social anxiety disorder reported higher ratings of craving for cigarettes as compared to non-smokers during abstinence. In addition, smokers with anxiety disorders are less responsive to nicotine cessation treatments, and they report that the reduction of stress is the main motivation for continued smoking (Battista et al., 2008; Evatt & Kassel, 2010; Piper et al., 2010). As a result, people with anxiety disorders experience more difficulty quitting smoking relative to smokers without anxiety disorders (Piper et al., 2011; Leyro et al., 2008; Watson, 2012; Zvolensky et al., 2004). In summary, there is evidence to suggest that people with anxiety disorders are more likely to smoke and experience greater withdrawal during smoking abstinence.

Women with anxiety disorders display higher rates of smoking than men: Clinical reports have shown that the prevalence of anxiety disorders and smoking behavior is higher among women as compared to men. A large population survey showed that there is a stronger co-morbid association between anxiety disorders and smoking rates in women as compared to men (Mykletun et al., 2008). In addition, a longitudinal study showed that women with a prior history of an anxiety disorder are more likely to develop tobacco dependence later in life relative to men (Brook et al., 2012). Stewart et al. (1997) showed that women who are hyper-sensitive to anxiety are more likely to engage in cigarette use to cope with anxiety as compared to males. In addition, John et al. (2004) demonstrated that women have a greater likelihood of developing anxiety disorders after a year of smoking as compared to men. Taken together, these studies suggest that there is a strong relationship between anxiety disorders and smoking, especially among women.

Heredity factors and smoking vulnerability: There is strong evidence that familial history of cigarette use is associated with heighten stress responses and a greater vulnerability to tobacco use. For example, Scherrer et al. (2012) showed that individuals with a family history of cigarette use are more likely to become smokers and report relaxation as the main motive for smoking as compared to smokers without a family history of cigarette use. Perkins et al. (2009) also demonstrated that individuals with a familial history of cigarette use reported stronger craving for nicotine nasal spray as compared to individuals with non-smoking parents. In addition, Erblich et al. (2003) demonstrated that smokers with a family history of cigarette use displayed higher ratings of craving when exposed to a mild stressor versus smokers without a family history of cigarette use. Brooks et al. (2008) also showed that smokers who have parents who smoke report a stronger craving for cigarettes after a mild stressor as compared to smokers who have non-smoking parents.

Several studies have also examined the genetic and environmental contribution to smoking behavior amongst monozygotic (MZ) and dizygotic (DZ) twins. These studies suggest that having a twin who smokes is the strongest predictor of nicotine dependence (Kendler et al., 1999; Maes et al., 1999; Sullivan & Kendler, 1999; Swan et al., 1997). These findings are further supported by more recent studies suggesting that the strongest predictor of smoking behavior among twins is genetic as compared to environmental factors including peer influence or socioeconomic status (Lessov-Schlagger et al., 2006; Meas et al., 2004; Munafo & Johnsone, 2008; Zavos et al., 2012).

Heredity factors promote smoking in women: Women appear to be more vulnerable to tobacco use when considering heredity factors. For example, Colamussi et al. (2007) showed that women with first-degree relatives who smoke displayed higher levels of stress-induced cigarette craving as compared to their male counterparts. In addition, a meta-analysis of 17 studies found that in comparison to males, females with a twin who smokes are more likely to engage in smoking behavior (Li et al., 2003). Although sociocultural factors play a role in smoking initiation among female twins (Hamilton et al., 2006), nicotine dependence has been mostly attributed to hereditary and genetic factors among female twins (Kendler et al., 1999). These studies suggest that there is a stronger relationship between heredity factors and vulnerability to smoking in women as compared to men.

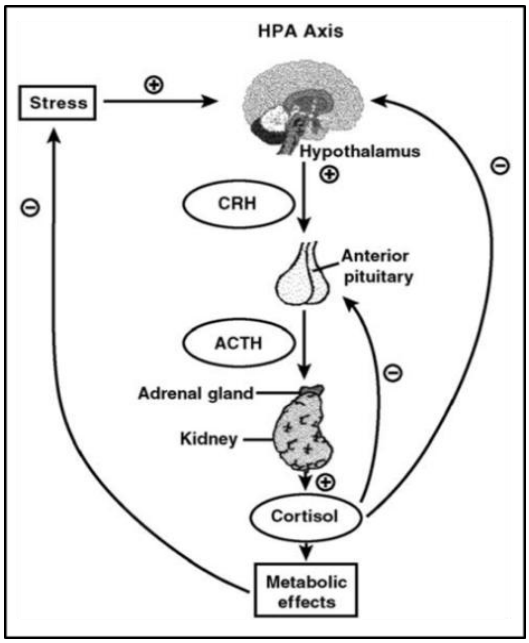
Summary: Taken together, there is clinical evidence that females have a predisposition to tobacco use due to a hypersensitive response to stress during nicotine withdrawal. However, there is a lack of pre-clinical studies examining whether the underlying substrates of stress are different between adult males versus females during nicotine withdrawal. Furthermore, it is unclear whether these potential sex differences to stress produced by withdrawal exist as early as

adolescence. To address the hypothesis that there are sex differences to stress during nicotine withdrawal, this dissertation compared anxiety-like behavior and biological markers of stress in adult and adolescent male and female rats during withdrawal from nicotine.

1.6 Underlying neurobiology of brain reward pathways and stress systems

Brain reward pathways: The mesolimbic dopamine pathway is believed to modulate positive affective responses including eating palatable foods, exercise, and consumption of drugs of abuse (see Kalivas & Volkow, 2005; Nestler, 2005). The mesolimbic pathway consists of neuronal projections that originate in the ventral tegmental area (VTA) and project to forebrain structures including the amygdala and nucleus accumbens (NAcc) (Aston-Jones & Harris, 2004; Carelli & Wightman, 2004; Koob & Volkow, 2010). There are other major dopaminergic pathways such as the nigrostriatal, mesocortical and tuberoinfundibular pathways (Albanese et al., 1986; Arias-Carrion et al., 2010). However; much research has shown that nicotine exerts rewarding effects via activation of neural circuits in the mesolimbic dopamine pathway. Specifically, nicotine binds to nicotinic acetylcholine receptors (nAChRs) localized within the VTA (Mansvelder & McGehee, 2002; Xu et al., 2006). The nAChRs are anatomically positioned to alter dopamine transmission by stimulation of voltage-sensitive calcium channels on glutamatergic neuronal terminals (Azam et al., 2007; McGehee & Role, 1995; Pidoplichko et al., 2004; Rathouz et al., 1996; Nomikos et al., 2000). The influx of calcium produces a release of the excitatory neurotransmitter glutamate which stimulates dopamine release in the NAcc (Gao et al., 2010; Mao et al., 2011). Behavioral work has demonstrated that nicotine reward is mediated, in large part, by increasing dopamine levels in the NAcc (Balfour, 2002; Mansvelder et al., 2003). Interestingly, the neurochemical effects of nicotine are reversed during nicotine withdrawal such that NAcc dopamine levels are decreased (Carboni et al., 2000; Di Chiara et al.,

2000; Hildebrand et al., 1998; Rada et al., 2001). Thus, dopamine in the NAcc is critically important to both reward and withdrawal.

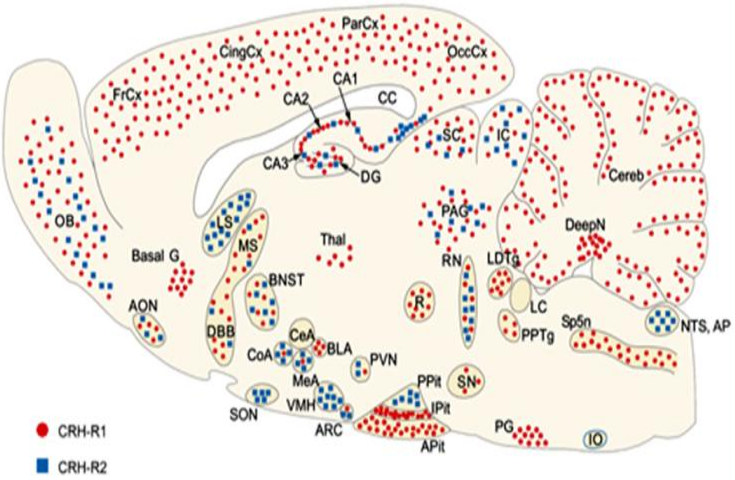


The biology of the stress response: The stress response is elicited in response to an adverse stimulus that causes a disruption of homeostasis (for a review, see Sawchenko et al., 1993). The main neuroendocrine substrate of the stress response is the hypothalamic-pituitary-adrenal (HPA) axis (Vale et al., 1981). The illustration to the right depicts physiological substrates of the HPA axis (adapted from Ryan & Thakore, 2002).

The HPA axis is a major physiological system that includes an interaction of the brain (hypothalamus) and the systemic (adrenal cortex) systems (Dunn & Berridge, 1990; De Souza & Grigoriadis, 2002; Vale et al., 1981; Ziegler & Herman, 2002). When a stressor is experienced, corticotropin releasing hormone (CRH) is secreted from the hypothalamus. CRH is 41-amino acid polypeptide that is primarily synthesized in the paraventricular nucleus (PVN) of the hypothalamus (Dunn & Berridge, 1990; Olschowka et al., 1982). The release of CRH then stimulates adrenocorticotropic hormone (ACTH) release from the anterior pituitary gland (Olschowka et al., 1982; Sawchenko et al., 1993; Semba et al., 2004). ACTH then simulates the release of corticosterone (a stress marker in rodents that is analogous to cortisol in humans) and other glucocorticoids from the adrenal cortex, which is located in the periphery area of the kidneys (Semba et al., 2004). Corticosterone released by the adrenal cortex circulates via the blood stream, and serves as a major negative feedback that terminates HPA axis activity (Keller-

Wood & Dallman, 1984). The action of corticosterone to inhibit CRH release is mediated directly at the level of the PVN via activation of glucocorticoid receptors (Liposits & Paull, 1989; Olschowka et al., 1982; Sawchenko et al., 1992; Vale et al., 1981). Within hypothalamic PVN neurons, corticosterone binds to nuclear glucocorticoid receptor II subunits causing an inhibition of CRH mRNA synthesis (Tanimua & Watts, 1998). In addition to glucocorticoid receptors, mineralocorticoid receptors are also involved in maintaining basal HPA axis activation. It is also thought that corticosterone can inhibit hypothalamic CRH and ACTH secretion by binding to glucocorticoid receptors in the hippocampus, prefrontal cortex and pre-optic area (De Souza & Grigoriadis, 2002; Ziegler & Herman, 2002).

CRH systems in the brain: Although CRH plays a primary role in mediating the HPA axis via the hypothalamus, CRH systems have a wide distribution throughout the brain (for a review, see De Souza & Grigoriadis, 2002). The illustration to the right depicts the distribution of corticotropin releasing hormone receptor subtype 1 (CRH-1) and subtype 2 (CRH-2) in the brain (adapted from Steckler & Holsboer, 1999). To date, three different CRH binding sites have been found in mammals, these are the CRH-1, and the CRH-2 receptor, and the CRH-binding protein complex (CRH-BP) (Kudielka & Kirschbaum, 2005; Sarnyai et al., 2001; Steckler & Holsboer, 1999).



Kudielka & Kirschbaum, 2005). When CRH binds to these receptors, it activates adenylate cyclase to convert adenosine triphosphate into cyclic adenosine monophosphate (cAMP), a second messenger that is involved in intracellular transmission. These receptors are found in several brain regions including the cerebral cortex, cerebellum, raphe nucleus, NAcc, hippocampus, amygdala, and superior colliculus (Aguilera et al., 2004; Bittencourt & Sawchenko, 2000; Bruijnzeel & Gold, 2005; Hsu et al., 2009; Valentino et al., 2010). The CRH-BP is a binding complex with the highest affinity for CRH as compared to CRH-1 and CRH-2 receptors. CRH-BP is found in the amygdala, bed nucleus of the stria terminalis, NAcc, preoptic nucleus, hippocampus, neocortex, olfactory bulb, and pituitary gland (Bruijnzeel & Gold, 2005; Potter et al., 1992; Kemp et al., 1998). Within the pituitary gland, it is believed that CRH-BP regulates HPA axis activation by acting as a competitive binding site that counteracts the effects of CRH-1 receptor activation by preventing the release of ACTH (Kemp et al., 1998).

Although CRH is primarily synthesized within the PVN of the hypothalamus, CRH also has a wide distribution throughout the brain. For example, CRH is distributed throughout the neocortex, periaqueductal gray, olfactory bulb, hippocampus, pons, raphe nucleus, bed nucleus of the stria terminalis, and NAcc shell (Hsu et al., 2009; Lim et al., 2007; Olschowka et al., 1982; Palkovits et al., 1992; Sarnyai et al., 2001; Sawchenko et al., 1993). In addition, CRH producing neurons have been found in the substantia nigra, VTA, medulla oblongata, central nucleus of the amygdala, and locus coeruleus (LC) (Caberlotto et al., 2004; Olschowka et al., 1982; Sarnyai et al., 2001; Ungless et al., 2003; 2010).

The role of CRH in extra-hypothalamic brain regions is largely unclear. However, several studies have found evidence to suggest a modulatory role of CRH on neurotransmission (Makino et al., 2002). For example, the CRH system is believed to alter gamma-aminobutyric acid

(GABA) transmission. This is supported by findings showing that CRH synthesis and storage is found within GABAergic neurons of the hippocampus, locus coeruleus and amygdala (Gallagher et al., 2008; Ungless et al., 2010; Valentino et al., 1983). In addition, Nie et al. (2004) showed that CRH administration in mice enhances GABAergic neurotransmission in the central nucleus of the amygdala. CRH-R1 receptors have also been shown to elevate intracellular calcium levels in neurons, whereas CRH-BP inhibits this effect (Van de Eede et al., 2005). Anatomical data also suggests that CRH may facilitate dopamine transmission. This possibility is supported by findings showing that CRH (Almela et al., 2012; Lemos et al., 2012; Swanson et al., 1983), CRH producing neurons (Ungless et al., 2010), and CRH receptor subtypes (Potter et al., 1994; Ungless et al., 2003; Van Pett et al., 2000) are found within regions of the mesolimbic dopamine pathway. Collectively, these findings suggest that the CRH system may modulate neuronal transmission in extra-hypothalamic regions, including terminal regions of the mesolimbic pathway. However, there is still a critical knowledge gap regarding the role of CRH within these regions.

1.7 Rationale for assessing CRH mRNA in the NAcc, amygdala, and hypothalamus

NAcc: The NAcc plays a primary role in drug reward and withdrawal (Albanese et al., 1986; Aston-Jones & Harris, 2004; Carelli & Wightman, 2004; Koob, 2009b; Nader et al., 1992). A recent study demonstrated that the NAcc is also activated following restraint stress in rats, suggesting that this region also plays a direct role in stress responses (Noh et al., 2012). Furthermore CRH is highly concentrated in the most caudal portion of the NAcc shell (Swanson et al., 1983). In addition, there is evidence to suggest that stress responses are elicited in the NAcc during drug withdrawal. For example, systemic administration of a CRH receptor antagonist increases dopamine levels in the NAcc in rats (Gurkovskaya et al., 2005). In addition,

administration of a non-specific CRH receptor antagonist in the NAcc blocks the behavioral effects of nicotine withdrawal in rats (Marcinkiewcz et al., 2009). Taken together, these studies suggest that the NAcc is a structure involved in mediating stress responses during drug withdrawal.

Amygdala: Although the PVN is known as the main site for CRH production, the amygdala is another primary source for CRH synthesis (Gallagher et al., 2008; Nie et al., 2004; Olschowka et al., 1982). More specifically, within the central amygdala it has been shown that subdivisions of GABAergic neurons are able to synthesize CRH (Veinante et al., 1997). Several studies have demonstrated that the amygdala is a limbic structure closely linked to the stress response during withdrawal (Koob, 2009a; 2009b). For example, elevated levels of CRH have been found in the amygdala during withdrawal from ethanol (Funk et al., 2006; Merlo Pich et al., 1995), cocaine (Richter & Weiss, 1999), cannabinoids (Rodriguez de Fonseca et al., 1997), and nicotine (Aydin et al., 2011; George et al., 2007) in rats. In addition, several reports have demonstrated that the behavioral effects of ethanol and nicotine withdrawal are blocked via administration of a non-selective CRH receptor antagonist in the amygdala (Bruijnzeel et al., 2012; Funk et al., 2006; Funk & Koob, 2007; Marcinkiewcz et al., 2009). These studies suggest that the amygdala is involved with modulation of stress responses produced by drug withdrawal.

Hypothalamus: A major site for CRH synthesis and regulation of the stress response is the hypothalamus (Dunn & Berridge, 1990; De Souza & Grigoriadis, 2002; Ziegler & Herman, 2002). Given the primary role of the hypothalamus in modulating the HPA axis, this dissertation also includes an analysis of this region as a positive control that may likely reveal changes in responses to withdrawal from nicotine.

1.8 Quantification of CRH mRNA

Evidence of CRH synthesis within the brain can be achieved by quantitative reverse-transcription polymerase chain reaction (qRT-PCR). In this procedure, mRNA is isolated and purified from neuronal tissue samples (Wong & Medrano, 2005). The purified RNA strands are then reverse transcribed into their complementary DNA (cNDA) strands by using the enzyme reverse transcriptase (Arikawa et al., 2008; Bustin et al., 2005). The resultant cDNA strands are then coupled with regions of nucleic acid strands called probes or primers, which are specific short DNA sequences for the gene of interest. The sample cDNA and specific primers are then mixed with SYBR green, a specific nucleic acid dye that fluoresces with the detection of target genes (Arya et al., 2005; Smith & Osborn, 2009). Amplification of the target gene is then achieved using conventional polymerase chain reaction techniques via a series of thermo-cycling steps involving high temperatures to denature and separate cDNA strands. This allows for primers to attach and signal specific complementary sequences (Arikawa et al., 2008; Wong & Medrano, 2005). Thus, the strength of qRT-PCR methods is the ability to detect and amplify specific DNA sequences with a high level of sensitivity for quantifying gene expression via mRNA production (Smith & Osborn, 2009).

By examining gene expression of CRH in extra-hypothalamic brain regions, a better understanding of the biological processes that occurs in response to stress can be achieved at the molecular level. This is important because quantification of CRH gene expression can be detected via CRH mRNA production. Furthermore, the accumulation of mRNA product in response to an environmental manipulation reflects a pre-translational step (Livak et al., 2001; Yuan et al., 2008). A pre-translational change suggests that at the cellular level, specific

mechanisms are activated to promote the expression of target proteins and/or hormones. Thus, assessment of CRH mRNA product is important to the goal of this research, as changes in CRH mRNA would suggest activation of CRH stress systems.

1.9 Rodent models of nicotine dependence and withdrawal

Nicotine dependence induction: The most commonly used method of inducing nicotine dependence in rodents is via surgical implantation of osmotic pumps that continuously deliver nicotine (for a review, see Malin & Goyarzu, 2009). In this model, osmotic pumps containing nicotine are surgically placed in the subcutaneous layer of a rodents' back. Once inside the subcutaneous layer, nicotine is released at a constant rate of 3 to 9 mg/kg per day (Hamilton et al., 2010; 2009; Kolokotroni et al., 2012; Malin et al., 2001; 2009; O'Dell et al., 2006; 2007). In order to ensure the development of nicotine dependence, nicotine is delivered for at least 7 days in rats (Besheer & Bevins, 2003; Malin et al., 1992; Matta et al., 2006; O'Dell et al., 2004; 2007; O'Dell & Khroyan, 2009; Suzuki et al., 1996; 1999; Wilmouth & Spear, 2006). This dosing regimen is clinically relevant, as humans consume on average five to six cigarettes a day which contain approximately 1.5 mg of nicotine each (Hukkanen et al., 2005; Matta et al., 2007).

Nicotine withdrawal induction: Osmotic pumps have been widely used to induce a reliable and well-documented withdrawal syndrome in rodents (for a review, see Malin & Goyarzu, 2009). Withdrawal is induced following the removal of the nicotine pump (spontaneous withdrawal) or administration of nicotinic receptor antagonist (precipitated withdrawal). In either method, nicotine withdrawal produces a behavioral profile involving physical signs including, writhes, gasps, shakes, tremors, teeth chattering, chewing, and ptosis (Hamilton et al., 2009; 2010; Malin et al., 1992; Malin & Goyarzu, 2009; Skjei & Markou, 2003). Nicotine withdrawal has also been shown to produce an increase in negative affective

states. For example, animals experiencing nicotine withdrawal avoid a compartment that was previously paired with nicotine withdrawal, as assessed by conditioning procedures (Bruijnzeel, 2012; for a review, see O'Dell & Khroyan, 2009). The nature of these negative affective states also involves the induction of stress systems, as nicotine withdrawal has been shown to induce anxiety-like behavior.

Behavioral assessment of anxiety-like behavior produced by withdrawal: There are several behavioral tools that are used to model anxiety-like behavior in rodents. The most widely used and accepted behavioral tools to measure anxiety-like behavior in rodents are the elevated plus maze and open field tests. The elevated plus maze consists of a four-armed maze that is elevated two feet from the ground. Two of the arms on the plus maze are surrounded by tall hallways that enclose arms of the maze. The other two arms are open platforms with no protective walls (Bourin et al., 2007). The elevated plus maze takes advantage of a rodent's natural tendency to avoid high risk areas such as the elevated and open hallways. Thus, rodents naturally prefer the enclosed areas. The closed arms are perceived to be a safer location relative to the open arms for a rodent that is averse to open spaces to avoid predators. The amount of time spent in the open and closed arms is recorded simultaneously for five minutes (Walf & Frye, 2007). When rodents are experiencing stress, they will spend even more time in the enclosed arms as compared to a control animal. A rodent which is not experiencing stress will engage in more exploratory behavior and spend more time in the open arms (Overstreet, 2012).

Similarly to the elevated plus maze, the open field test consists of a squared open area surrounded by four walls. The top of the open field is covered by a transparent top that prevents rodents from leaving the box. The bottom of the open field is marked by a grid floor so that time spent in each area of the open field can be recorded. The open field test also takes advantage of a

rodent's natural tendency to avoid open exposed areas. Thus, rats prefer the corners of the open field as compared to the center (Bourin et al., 2007; De Boer & Koolhaas, 2003). When rodents are stressed, they will spend more time in the periphery and corner areas of the open field as these areas are perceived to be a safer location relative to the center. When rodents are not experiencing stress, they will spend more time exploring the center area (Overstreet, 2012). Other behavioral tools utilized to measure anxiety-like behaviors include the light/dark exploration test and the defensive burying test where latency to burying a shock probe is recorded (Bourin et al., 2007; De Boer & Koolhaas, 2003).

1.10 Studies assessing anxiety-like behavior produced by nicotine withdrawal

Several laboratories have shown that rodents experiencing nicotine withdrawal elicit anxiety-like behavior (Bruijnzeel et al., 2012; O'Dell, 2009). For example, nicotine-dependent rats (Tejeda et al., 2012; Wilmouth & Spear, 2006) and mice (Damaj et al., 2003; Kota et al., 2008; Rehni et al., 2011) spend more time in the enclosed arms of the elevated plus maze during precipitated nicotine withdrawal. Similarly, several studies have also demonstrated that under spontaneous nicotine withdrawal conditions, rats (Jonkman et al., 2008; Irvine et al., 2001; Chae et al., 2007) and mice (Damaj et al., 2003; Kota et al., 2007; Stoker et al., 2008) display anxiety-like behavior on the elevated plus maze. Furthermore, nicotine withdrawal also produces anxiety-like behavior on the open field test, as rodents spend more time in the periphery areas of the open field (Tzavara et al., 2002). Additionally, withdrawal from nicotine produces anxiety-like behavior in rats as assessed by the defensive burying paradigm (George et al., 2007), light/dark exploration test (Stoker et al., 2008; Jonkman et al., 2005) and startle-response test (Helton et al., 1993). Collectively, these studies demonstrate that during nicotine withdrawal, rats and mice display anxiety-like behavior.

Numerous studies have reported anxiety-like behavior in rats and mice 24 hours after nicotine pump removal (Chae et al., 2007; Jonkman et al., 2005; Irvine, 2001; Stoker et al., 2008). Furthermore, Jonkman et al. (2008) also showed that 24 hours after nicotine pump removal, mice display greater anxiety-like behavior compared to mice under pharmacologically precipitated withdrawal conditions. In addition, Skjei and Markou (2003) and Shram et al. (2008) showed that in rats, somatic signs of nicotine withdrawal are highest 24 hours after nicotine pump removal compared to later time points. Taken together, these studies suggest that under spontaneous withdrawal conditions, anxiety-like behaviors and nicotine withdrawal are most robust 24 hours after pump removal. Thus, this dissertation employs these parameters by examining anxiety-like behaviors in adult and adolescent male and female rats 24 hours after nicotine pump removal.

1.11 Biological markers of stress are activated during nicotine exposure and withdrawal

Nicotine exposure is known to elicit biological markers of stress in rodents. For example, moderate to high doses of nicotine (0.5 – 2.0 mg/kg) have been shown to increase plasma ACTH and corticosterone levels in rats (Gadek-Michalsk & BugaJski, 2004; Lufty et al., 2006; 2012; Moidel et al., 2006; Porcu et al., 2003; Rhodes et al., 2001a; 2001b; Skwara et al., 2012). Nicotine via intravenous administration also increases plasma ACTH levels in rats (Matta et al., 1987; 1997).

In addition to nicotine being a stressor, withdrawal from chronic administration of this drug also increases biological markers of stress. Withdrawal from nicotine produces an increase in corticosterone, ACTH, and CRH release in rodents. For example, normal blood corticosterone levels in rats range between 80 and 200 ng/ml (Gentile et al., 2011; Mantch et al., 2007; Moidel et al., 2006; Pentkowski et al., 2011; Rhodes et al., 2001a; 2004; Semba et al., 2004). However,

during nicotine withdrawal, blood corticosterone levels increase between 500 and 800 ng/ml in rats (Gentile et al., 2011; Semba et al., 2004). Similarly, normal circulating blood ACTH levels in rats range between 30 and 50 pg/ml (Gentile et al., 2011; Chen et al., 2008; Moidel et al., 2006; Rhodes et al., 2001a; 2001b) and mice (Lutfy et al., 2006). However, in response to nicotine withdrawal, ACTH levels increase to a range between 200 and 500 pg/ml in rats (Gentile et al., 2011; Rhodes et al., 2004; Skwara et al., 2012) and mice (Lutfy et al., 2006).

In addition to ACTH and corticosterone, central brain changes in CRH systems have also been studied in rodents experiencing nicotine withdrawal. Aydin et al. (2011) showed that CRH mRNA levels are over-expressed in the central nucleus of the amygdala when nicotine-dependent rats are experience withdrawal. Consistent with this, George et al. (2007) demonstrated that nicotine-dependent rats display robust increases in CRH-like immunoreactivity in the amygdala during precipitated nicotine withdrawal. Furthermore, the latter study also demonstrated that intraventricular injections of a CRH-R1 receptor antagonist block nicotine intake in rats after a deprivation period from nicotine intravenous self-administration (IVSA) procedures.

Changes in CRH systems have also been studied during nicotine withdrawal by utilizing intracranial self-stimulation (ICSS) procedures during nicotine withdrawal. In this model, rats are first implanted with an electrode that is placed in the medial forebrain bundle, which consists of dopaminergic efferent projections to brain reward structures. Thus, animals readily perform operant responses for stimulation of this region. Rats are then allowed to self-administer electrical current to their brains in order to receive brain reward stimulation. Administration of nicotine lowers the electrical current threshold because of the rewarding properties of this drug. However, nicotine withdrawal increases brain reward thresholds because of a decrease in brain

reward produced by withdrawal (for a review, see Bauzo & Bruijnzeel, 2012). Studies using ICSS procedures have shown that intra-ventricular administration of nonspecific CRH-R1/R2 receptor antagonists reverses the deficits in brain reward function produced by nicotine withdrawal (Bruijnzeel et al., 2007; 2009). Furthermore, administration of non-specific CRH-R1/R2 receptor antagonists into the NAcc or amygdala also reverse the deficits in brain reward function produced by nicotine withdrawal (Bruijnzeel et al., 2012; Marcinkiewcz et al., 2009).

Summary: There is evidence to suggest that CRH in the NAcc and amygdala is involved in the modulation of nicotine withdrawal. Collectively, these reports demonstrate that nicotine exposure and withdrawal increase biological markers of stress in rodents. Thus, this dissertation examined corticosterone and CRH mRNA levels in the hypothalamus, amygdala, and NAcc of adult, adolescent male and female rats during nicotine exposure and withdrawal.

1.12 Sex differences in biological markers of stress during nicotine exposure and withdrawal

Several studies have suggested that females are more sensitive to the effects of nicotine exposure on HPA axis activation. Nicotine exposure increases CRH and ACTH levels to a greater extent in the hypothalamus of females versus males (Mcklveen et al., 2010; Moidel et al., 2006). In addition, nicotine administration via subcutaneous injection also increases plasma ACTH and corticosterone levels to a greater extent in female versus male rats (Gentile et al., 2011; Moidel et al., 2006; Rhodes et al., 2001a; 2004; Skwara et al., 2012). Furthermore, continuous infusion of nicotine via osmotic pumps also increases plasma ACTH and corticosterone levels to a greater extent in female versus male rats (Faraday et al., 2005). Exposure to nicotine via drinking water has also been shown to produce sex-dependent differences in responses to stress in mice. Specifically, female mice display more anxiety-like

behavior compared to their male counterparts (Caldarone et al., 2008). In summary, these studies suggest that nicotine exposure elicits greater stress and anxiety in female versus male rodents.

Only a limited number of studies have compared the effects of nicotine withdrawal across male and female rats. First, female adult rats display more physical signs of nicotine withdrawal relative to male rats (Hamilton et al., 2009). A subsequent report by the same group showed that there are no sex differences in physical signs of nicotine withdrawal between adolescent male and female rats (Hamilton et al., 2010). The latter finding may not be surprising given that adolescent rats display reduced effects of nicotine withdrawal as described below. Second, a recent report demonstrated that female adult rats display elevated plasma ACTH and corticosterone levels during nicotine withdrawal relative to their male counterparts (Gentile et al., 2011). Consistent with these findings, Skwara et al. (2012) also demonstrated that plasma ACTH and corticosterone levels are increased in female rats following precipitated nicotine withdrawal as compared to males. These studies suggest that nicotine withdrawal elicits greater stress responses in female versus male rats.

1.13 Age group differences to nicotine withdrawal in rodents

Much research has suggested that nicotine withdrawal is lower during the adolescent period of development in rodents (for a review, see O'Dell, 2009). For example, previous work from our laboratory and others has demonstrated that adult rats and mice display more physical signs of nicotine withdrawal relative to adolescents (Kota et al., 2007; 2008; Natividad et al., 2010; 2012; O'Dell et al., 2004; 2006; 2007; Shram et al., 2006; 2008). In addition, previous studies from our laboratory and others have also demonstrated that adult rats display more aversion for an environment previously paired with nicotine withdrawal as compared to adolescent rats (O'Dell et al., 2007; Shram et al., 2008; Tejeda et al., 2012). Adult rats also

display decreases in ICSS brain reward function during nicotine withdrawal, an effect that was not observed in adolescent rats (O'Dell et al., 2006). With regard to age differences in anxiety produced by nicotine withdrawal, adult rats and mice display an increase in anxiety-like behavior during nicotine withdrawal as compared to adolescents on the elevated plus maze (Kota et al., 2007; 2008; Tejeda et al., 2012; Wilmouth & Spear, 2006) and open field (Smith et al., 2006) tests. Neurochemical studies from our laboratory have also revealed that adult rats display a larger decrease in extracellular levels of dopamine in the NAcc during nicotine withdrawal as compared to adolescents (Natividad et al., 2010). Furthermore, a recent study from our laboratory demonstrated that adolescent rats were less sensitive to amino acid regulation of NAcc dopamine produced by nicotine withdrawal as compared to adults (Natividad et al., 2012).

Although there is much work showing that nicotine withdrawal is lower during adolescence, few studies have examined sex differences in nicotine withdrawal in adolescent rats. An assessment of sex differences to nicotine withdrawal during adolescence can be highly informative, as the reproductive hormonal systems that modulate sex differences in adulthood are not yet fully developed. Thus, this dissertation also includes an examination of sex differences in stress and anxiety during nicotine withdrawal in adolescent male and female rats.

1.14 Significance, Aims, and hypotheses of this dissertation

Significance: Tobacco use is a major health and economic concern. Despite the magnitude of this problem, there is still a critical knowledge gap in the understanding of how stress and anxiety contribute to enhanced tobacco use in women. While a variety of environmental and genetic factors contribute to the risk for nicotine addiction in women, the underlying substrates of this phenomenon are unclear. In addition, most smoking cessation strategies focus on alleviating withdrawal with limited success rates among women smokers.

Few pre-clinical studies have examined stress produced by nicotine exposure and withdrawal. Thus, this work will contribute novel information to the literature regarding sex and age differences to stress produced by nicotine exposure and withdrawal. Lastly, to our knowledge no one has characterized CRH gene expression during nicotine exposure and withdrawal in adult and adolescent male and female rats. This research is significant because these findings may provide an important first step toward understanding how stress and anxiety contribute to enhanced vulnerability to tobacco use among females. Furthermore, these studies may inform the manner in which individualized smoking cessation treatments can be developed for smokers of different age and sex classifications.

Aims: This dissertation compared behavioral indices of anxiety and biological markers of stress in two specific aims. In Aim 1, this dissertation examined whether *adult* male and female rats display sex-dependent differences in biological markers of stress and behavioral indices of anxiety during nicotine withdrawal. More specifically, anxiety-like behavior was examined on the elevated plus maze and open field tests, plasma corticosterone levels and changes in CRH gene expression in the amygdala, NAcc and hypothalamus were also explored. In Aim 2, this dissertation examined whether *adolescent* male and female rats display sex-dependent differences in biological markers of stress and behavioral indices of anxiety during nicotine withdrawal. In order to differentiate the effects of nicotine exposure vs. nicotine withdrawal, separate groups of adult and adolescent rats were tested with nicotine on board.

Previous work in our laboratory and others utilized the Alzet osmotic pumps to examine the behavioral and neurochemcial effects of nicotine withdrawal. However, it is unclear whether any systematic differences exist in the metabolism of nicotine across sex and age when employing these procedures. Thus, an initial study was conducted to determine equivalent

nicotine doses in female and male rats of different ages. This was achieved by administering various doses of nicotine in osmotic pumps, and comparing cotinine (a major metabolite of nicotine) levels at various time points during nicotine exposure and withdrawal.

Specific Aims of dissertation:

Initial studies to characterize equivalent doses of nicotine across age and sex

Aim 1: Characterize anxiety-like behavior and biological indices of stress during nicotine withdrawal in *adult* male and female rats.

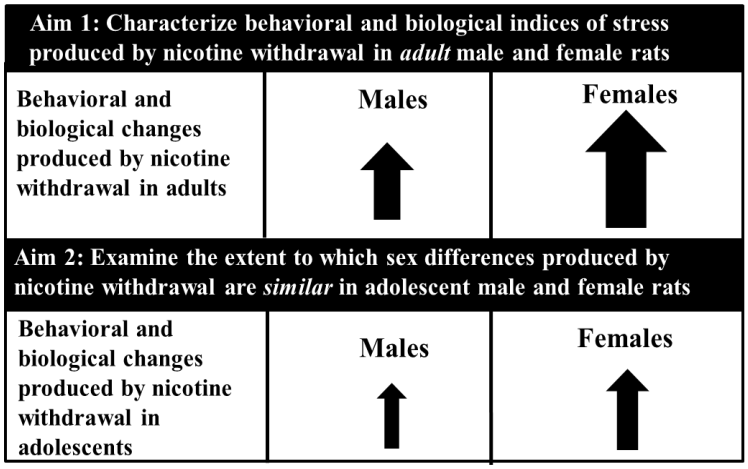
Aim 2: Examine the extent to which sex differences to anxiety-like behavior and biological indices of stress during nicotine withdrawal are similar in *adolescent* male and female rats.

Hypothesis for Aim 1: It was hypothesized that during nicotine withdrawal, adult female rats would display an increase in anxiety-like behavior and physiological markers of stress as compared to males. This hypothesis is based on clinical observations that women experience greater anxiety during tobacco cessation relative to men, as previously discussed.

Hypotheses for Aim 2: The first hypothesis for Aim 2 was that adolescent rats would display lower anxiety-like behavior and physiological markers of stress during nicotine withdrawal as compared to adults. This hypothesis is based on pre-clinical data from our laboratory and others showing that the aversive effects of nicotine withdrawal are lower during the adolescent period of development. The second hypothesis for Aim 2 was that during nicotine withdrawal, adolescent female rats would display increased anxiety-like behavior and physiological markers of stress relative to their adolescent male counterparts. This hypothesis is based on clinical data observations that adolescent females are more vulnerable to the negative aversive effects of withdrawal relative to adolescent males.

This illustration to the right depicts a schematic of the hypothesized results for specific Aims 1 and 2. The size of the arrows denotes the relative magnitude of the hypothesized changes for behavioral and biological markers of stress produced by nicotine withdrawal.

A schematic of the hypothesized results for Aim 1 and 2



Chapter 2: Methods

2.1 Initial study to determine equivalent doses of nicotine across age and sex

Prior to embarking on the dissertation studies, an initial study was conducted to determine equivalent nicotine plasma levels across experimental conditions. Nicotine metabolism was assessed indirectly by comparing cotinine (a nicotine metabolite) levels in plasma from adolescent and adult male and female rats during exposure and withdrawal from nicotine that was delivered via subcutaneous osmotic pumps. Adult rats received pumps that were appropriately sized for larger animals (4.5 mm in length; Alzet model 2ml2), whereas adolescents received either one or two pumps that were approximately half as small (2.5 mm in length; Alzet model 2002). Different doses of nicotine were delivered for 14 days, as described below. Plasma samples were collected from tail blood on days 7, 10, and 14 of nicotine exposure. After 14 days of nicotine exposure, the pumps were surgically removed and plasma samples were collected 6, 12, and 24 hours later. To avoid confounding stress produced by repeated tail vein blood sampling, separate groups of rats were used in these studies.

Four groups of rats were used to determine equivalent doses in adolescent and adult male and female rats. One group of adult rats (n=26) was prepared with pumps (model 2ml2) that delivered a nicotine dose of 3.2 mg/kg/day (expressed as base form) that produces robust physical and affective signs of withdrawal in adult rats (O'Dell et al., 2004; 2006). Given the fast growth rates and drug metabolism during adolescence, three groups of adolescent rats received pumps with different nicotine doses and experimental procedures. A group of adolescents (n=17) was prepared with one small pump (model 2002) that delivered 4.7 mg/kg/day of nicotine for 14 days. This dose was selected from previous work showing that adolescents implanted with a large pump (model 2ml2) require 1.5-fold higher doses of nicotine to produce equivalent levels

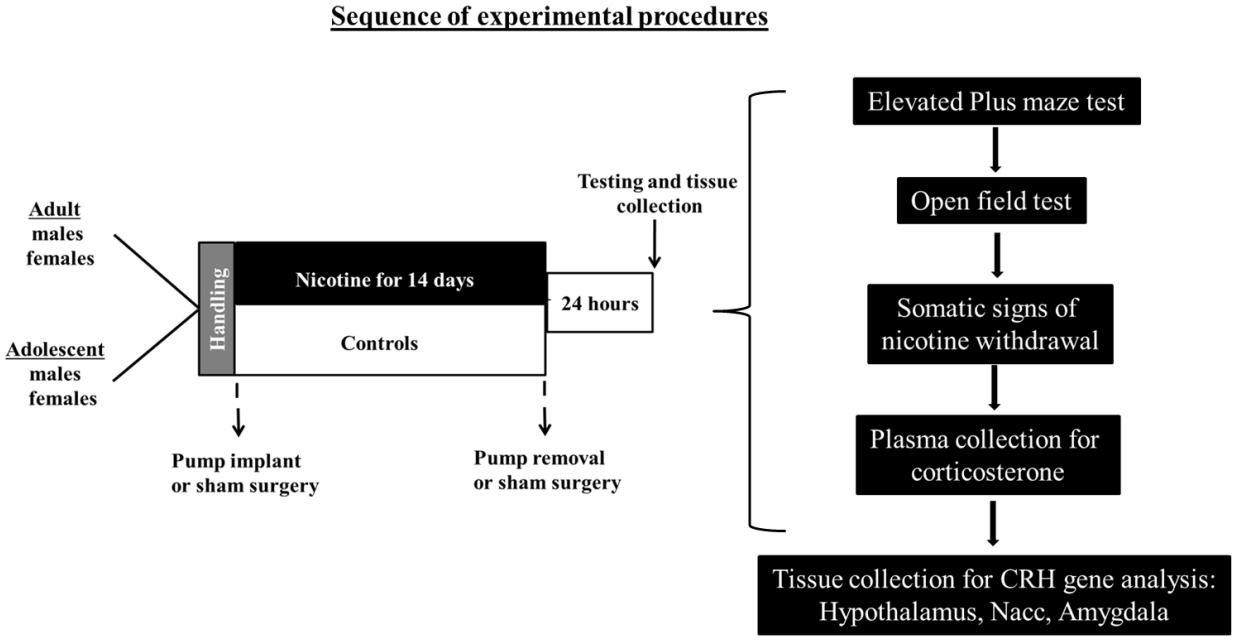
in adult rats (O'Dell et al., 2006). A second group of adolescents (n=17) was prepared with one small osmotic pump containing 4.7 mg/kg/day of nicotine. Seven days later, the pump was replaced with a new pump that was re-adjusted for the rats' rapid weight gain. Last, a third group of adolescents (n=32) was prepared with two small pumps that each delivered 4.7 mg/kg/day each of nicotine for 14 days. This group received a total of 9.4 mg/kg/day of nicotine.

Plasma cotinine levels were analyzed using commercially available 96-well plate ELISA kits (OraSure Technologies, Inc., Bethlehem, PA). Standard curves were used to estimate plasma cotinine levels using a Spectra Maxplus spectrophotometer (Molecular Devices Inc, Sunnyvale, CA). The data were analyzed using a three-way mixed factorial ANOVA. The analysis included sex (male or female) and treatment group (adults with one large pump, adolescents with one small pump, adolescent re-pumped, and adolescents with two small pumps) as between-subjects factors. Time point of sample collection (days during exposure or hours during withdrawal) was included as a within-subject factor.

The results from this study are shown in Figure 1. Regarding sex differences, the results revealed that there were no sex differences in cotinine levels during nicotine exposure [$F(1,79)=0.96$; p=ns] and withdrawal [$F(1,84)=0.19$; p=ns] regardless of the age of the animals. This suggests that sex differences can be appropriately compared across all of the nicotine pump conditions. Regarding age differences during nicotine exposure, adults displayed higher cotinine levels than adolescents prepared with one small pump and adolescents re-implanted with one small pump that was adjusted for weight gain (main effect of treatment $F(3,79)=8.96$; $p\leq0.05$). However, adult cotinine levels were similar to that of adolescents prepared with two small pumps that each delivered 4.7 mg/kg/day of nicotine for 14 days. A similar pattern was observed during nicotine withdrawal, such that similar levels of cotinine were observed in adults and

adolescents that were implanted with two small pumps that each delivered 4.7 mg/kg/day each of nicotine. These data suggest that adolescents require two osmotic pumps delivering a total nicotine volume of 9.4 mg/kg/day to produce similar cotinine levels as adults with one pump that delivers 3.2 mg/kg/day. *This study provided appropriate parameters to conduct the dissertation studies comparing sex and age differences in anxiety-like behavior and biological markers of stress during nicotine exposure and withdrawal as described below.*

2.2 Experimental procedures



This diagram shows the integrative approach of this dissertation that applies behavioral and molecular techniques to compare age and sex differences in stress systems produced by nicotine exposure and withdrawal. Briefly, adolescent and adult male and female rats received a sham surgery or were implanted with nicotine pumps. After 14 days of nicotine exposure, the pumps were removed to induce spontaneous withdrawal. Twenty-four hours after pump removal, behavioral tests were conducted to compare physical signs of withdrawal and anxiety-like behavior, using the elevated plus maze and open field tests. After behavioral testing, the brains

were removed and analyzed for CRH mRNA levels using qRT-PCR. Blood samples were also collected and analyzed for corticosterone, a blood marker of HPA axis activation. To determine whether any observed effects were produced by nicotine exposure and not withdrawal, separate cohorts of rats did *not* have their pumps removed and were tested with nicotine circulating in their system.

2.3 Subjects

Male and female adult (n=66) and adolescent (n=32) Wistar rats were used. Rats were bred in the Psychology Department from a stock of bred out Wistar rats from Harlan, Inc (Indianapolis, IN). All rats were housed in groups of two to three per cage in a humidity- and temperature-controlled (20-22^{o}C) vivarium using a 12-/12-hour light/dark cycle with lights off at 8:00 a.m. The home cages consisted of a rectangular Plexiglas® hanging cage (41.5 cm long x 17 cm wide x 21 cm high) with pine bedding. Rats had ad libitum access to standard rodent chow and water at all times except during testing. Adults were postnatal day (PND) 60 and adolescents were PND 28 at the start of the experiment. All rats were handled for approximately five minutes per day for three days prior to the start of experimentation. All procedures were approved by the UTEP Animal Care and Use Committee and followed the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.4 Behavioral tests

Elevated plus maze testing: Rats were tested for anxiety-like behavior using the elevated plus maze procedure. The animals were first acclimated to the testing room in a rectangular Plexiglas® cage for 20 minutes. After 20 minutes, the rats were placed onto the elevated plus maze, which was in the middle of the testing room beneath a red light. The plus maze apparatus consisted of four arms (10 x 50 cm) that were elevated to a height of 50 cm above the ground.

The closed arms had 40 cm high walls around them, and the open arms did not have walls that enclosed the open platforms. At the beginning of the test, the rats were placed into the maze facing the open arm and time spent in each arm was recorded for five minutes. The maze was thoroughly cleaned with 70 percent ethanol and then water between each individual test. Rats that fell off the maze were excluded from the study.

Open field test: After elevated plus maze testing, the rats were returned to the isolation cage for 10 minutes. The open-field apparatus consists of a clear Plexiglas® box (60 x 60 x 15 cm) that was positioned in the middle of an adjacent room beneath a red light. The walls of the maze were clear and the floor was divided into 25 equal squares (12 x 12 cm; 16 peripheral and 9 center squares). At the start of the test, rats were placed in the center of the open field, and time spent in the center versus corner areas was recorded for five minutes.

*Somatic signs of withdrawal testing***:** After the open field test, the rats were returned to the isolation cage. Ten minutes later, the rats were moved to another testing room and placed in a clear Plexiglas® cylindrical container (30 x 29 cm) cage for 10 minutes. Rats were then monitored for physical signs of nicotine withdrawal for 10 minutes. The observed signs include blinks, writhes, body shakes, teeth chatters, gasps, and ptosis. If present continuously, ptosis was only counted once. The total number of somatic signs was defined as the sum of individual occurrences of the aforementioned signs during the entire observation period.

2.5 Biological markers of stress

CRH gene expression procedure: After behavioral testing, rats were sacrificed by rapid decapitation to ensure preservation of the neurochemical environment and minimize degradation during tissue dissection. The amygdala, hypothalamus, and NAcc from both hemispheres were collected and flash frozen at -80 ºC within an estimated time of 30 seconds from sacrifice. Total

RNA was isolated from neuronal tissue samples using the All Prep DNA/RNA Mini kit (QIAGEN, Inc.) for small tissue sections. After isolation, RNA was quantified using a full-spectrum UV/V is spectrophotometer (Beckman Coulter Inc.). The target ratio of 1.8-2.0 for A260/280 was used as an inclusion criterion for all RNA samples. The quality of the RNA was then visualized by MOPS one percent agarose gel (37 percent formaldehyde) using the Thermo Scientific easy cast electrophoresis system. The gels were verified for characteristic 18S and 28S ribosomal RNA bands using ethidium bromide and the Bio-Rad ChemiDoc XRS+ imaging system. Samples that had insufficient amounts of RNA were excluded from further analyses.

One microgram of total RNA was then digested with DNaseI, Amp Grade (Invitrogen) prior to cDNA synthesis in order to remove any DNA contamination. The RNA was then reverse transcribed into cDNA with the Advantage® RT-for-PCR kit (Clontech) using Oligo(dT) primers, following the manufacturer's instructions. Once the cDNA was synthesized, the cDNA samples were diluted 1:10 in nuclease-free H_2O, separated into aliquots and stored at -20 ºC. Specific primers for CRH and reference gene RPL13A were obtained from Integrated DNA Technologies, Inc. with amplicons between 71 and 142 base-pairs.

The rationale for the reference gene is based upon an initial study examining tissue from a group of adult rats (n=27) that was conducted before quantifying CRH gene expression across experimental groups. Four commonly used reference genes were tested as potential candidates for the normalizing gene, including: actin (Actb), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), RNA polymerase II (Pol2a) and ribosomal protein L13A (Rpl13A). The findings revealed that the expression of Rpl13A was stable and similar across male and female control and nicotine-treated rats.

The remaining samples were analyzed using Rp113a as a reference gene. Commercially available SYBR® Fast qPCR fluorescent labeling kits (Kapa Biosystems, Inc.) were used to perform qRT-PCR using the Mastercycler ep Realplex2 System (Eppendorf, Inc.). All samples were analyzed in triplicates and amplified by the following protocol: initial denaturing at 95 ºC for five minutes, continued denaturing at 95 ºC for 15 seconds; annealing at 59 ºC for 15 seconds; extension at 68 ºC for 20 seconds, for a total of 40 cycles. CRH mRNA expression was normalized by RPl-13A mRNA expression using the comparative C_T method adopted from Schmittgen and Livak (2008). The amplification specificity for each primer was tested for a single-product, as shown by a single band via TAE one percent gel electrophoresis and visualized on the Bio-Rad ChemiDoc XRS+ system.

Corticosterone assay: Corticosterone levels were assessed in blood samples that were collected from trunk blood during sacrifice. The samples were centrifuged for 15 minutes at 5000 x g at 4 °C. The resultant plasma was then stored at -80 °C until analyzed. Corticosterone levels were estimated using a 96-well plate ELISA kit (Assaypro Inc.) using a Spectra Maxplus spectrophotometer (Molecular Devices Inc.).

2.6 Statistical approach

Each measure was analyzed using a three-way ANOVA with sex (male and female), age (adult and adolescent) and treatment (control, nicotine exposure, and nicotine withdrawal) as between subject factors. In cases where three-way interaction effects were significant, individual group comparisons were reported. However, in cases where three-way interactions were not significant, two-way interactions were reported. All post-hoc tests were conducted using Fisher's LSD tests where appropriate ($p \leq 0.05$).

Chapter 3: Results

3.1 Behavioral tests

*Somatic signs of withdrawal***:** Figure 2 illustrates somatic signs of withdrawal during nicotine exposure and withdrawal in adult and adolescent male and female rats. Somatic signs were analyzed using the total amount of signs elicited during the entire observation period. A three-way analysis of withdrawal signs revealed that there were no interaction effects between sex, age, and treatment [$F(2, 92)=0.84$; p=ns]. However, a two-way analysis of withdrawal signs revealed a significant interaction between age and treatment [$F(2, 92)=12.08$; $p\leq0.05$]. Subsequent post-hoc analyses revealed that adult rats that were tested during nicotine withdrawal displayed an increase in signs of withdrawal compared to their respective controls (*$p\leq0.05$). There were no differences in the magnitude of withdrawal signs across male and female adolescent rats.

Elevated plus maze: Figure 3 illustrates anxiety-like behavior as assessed by the elevated plus maze during nicotine exposure and withdrawal in adult and adolescent male and female rats. Anxiety-like behavior was operationally defined as an increase in time spent in the closed arm as compared to controls. Four rats that fell of the maze were excluded from the overall analysis due to incompletion of the test. A three-way analysis of percent time spent in the closed arm revealed a significant interaction between sex, age, and treatment [$F(2, 96)=8.85$; $p\leq0.05$]. Subsequent post-hoc analyses revealed that adult females that were tested during nicotine exposure displayed an increase in anxiety-like behavior relative to controls (*$p\leq0.05$). However, adult females tested during nicotine withdrawal displayed an increase in anxiety-like behavior that was significantly higher than their respective controls (*$p\leq0.05$), male counterparts (†$p\leq0.05$), and adolescent counterparts (#$p\leq0.05$). In adolescents, the males displayed the largest effects of nicotine

exposure and withdrawal on anxiety-like behavior as compared to respective controls (*$p \leq 0.05$), female counterparts (†$p \leq 0.05$), and adolescent counterparts (#$p \leq 0.05$).

Open field: Figure 4 illustrates anxiety-like behavior as assessed by the open field test during nicotine exposure and withdrawal in adult and adolescent male and female rats. Anxiety-like behavior was operationally defined as an increase in time spent in the corners of the open field as compared to controls. A three-way analysis of percent corner time revealed a significant interaction between sex, age, and treatment [$F(2, 92)=3.85$; $p \leq 0.05$]. Subsequent post-hoc analyses revealed that adult females tested during nicotine exposure displayed an increase in anxiety-like behavior relative to controls (*$p \leq 0.05$). However, adult females tested during nicotine withdrawal displayed an increase in anxiety-like behavior that was higher than respective controls (*$p \leq 0.05$) and their male counterparts (†$p \leq 0.05$). In adolescents, males tested during nicotine withdrawal displayed an increase in anxiety-like behavior relative to controls (*$p \leq 0.05$). Adolescent female controls displayed an increase in anxiety-like behavior relative to males (†$p \leq 0.05$) and their adult counterparts (#$p \leq 0.05$).

3.2 Biological markers of stress

Plasma corticosterone levels: Figure 5 illustrates plasma corticosterone levels during nicotine exposure and withdrawal in adult and adolescent male and female rats. Eight rats were excluded from the overall analysis due to non-detectable signals of plasma corticosterone. A three-way analysis of corticosterone levels revealed a significant interaction between sex, age, and treatment [$F(2, 66)=3.2$; $p \leq 0.05$]. Subsequent post-hoc analyses revealed that adult males tested during nicotine exposure displayed an increase in corticosterone levels relative to controls (*$p \leq 0.05$). Adult females tested during nicotine withdrawal displayed an increase in corticosterone levels relative to controls (*$p \leq 0.05$), male counterparts (†$p \leq 0.05$) and adolescent

counterparts (#$p \leq 0.05$). In adolescents, the male controls and males tested during nicotine withdrawal displayed an increase in corticosterone levels relative to their adult counterparts (#$p \leq 0.05$).

CRH gene expression in the NAcc: Figure 6 illustrates CRH gene expression in the NAcc during nicotine exposure and withdrawal in adult and adolescent male and female rats. Four rats were excluded from the overall analysis due to insufficient RNA levels. A three-way analysis of CRH gene expression revealed a significant interaction between sex, age, and treatment in this brain region [$F(2, 42)=4.34$; $p \leq 0.05$]. Subsequent post-hoc analyses revealed that adult females tested during nicotine withdrawal displayed an increase in CRH gene expression relative to controls (*$p \leq 0.05$), male counterparts (†$p \leq 0.05$), and adolescent counterparts (#$p \leq 0.05$). In adolescents, females tested during nicotine withdrawal displayed a decrease in CRH gene expression relative to controls (*$p \leq 0.05$).

CRH gene expression in the amygdala: Figure 7 illustrates CRH gene expression in the amygdala during nicotine exposure and withdrawal in adult and adolescent male and female rats. Ten rats were excluded from the overall analysis due to insufficient RNA levels. A three-way analysis of CRH gene expression revealed that there were no interaction effects between sex, age, and treatment in this brain region [$F(2, 52)=0.21$; p=ns]. However, a two-way analysis of CRH gene expression in the amygdala revealed a significant interaction between sex and treatment [$F(2, 52)=3.72$; $p<0.05$]. Subsequent post-hoc analyses revealed that adult and adolescent male rats tested during nicotine exposure displayed a significant increase in CRH gene expression as compared to controls (*$p<0.05$) and female counterparts (†$p<0.05$). In addition, adolescent males tested during nicotine withdrawal displayed an increase in CRH gene expression relative to controls (*$p \leq 0.05$).

CRH gene expression in the hypothalamus: Figure 8 illustrates CRH gene expression in the hypothalamus during nicotine exposure and withdrawal in adult and adolescent male and female rats. Three rats were excluded from the overall analysis due to insufficient RNA levels. A three-way analysis of CRH gene expression revealed that there were no interaction effects between sex, age, and treatment in this brain region [$F(2, 71)=1.20$; p=ns]. However, a two-way analysis of CRH gene expression in the hypothalamus revealed a significant interaction between sex and treatment [$F(2, 71)=3.72$; $p<0.05$]. Subsequent post-hoc analyses revealed that adolescent males tested during nicotine exposure displaying a significant increase in CRH gene expression as compared to controls ($*p\leq0.05$).

Chapter 4: Discussion

4.1 Summary

The major finding of this dissertation is that female adult rats displayed significantly greater behavioral and biochemical indices of stress produced by nicotine withdrawal as compared to males. Specifically, adult females displayed enhanced anxiety-like behavior in the elevated-plus maze and open-field tests during withdrawal compared to males. Female adults also displayed an increase in plasma corticosterone levels that was highly correlated with anxiety-like behavior produced by withdrawal. Adult females also displayed a large increase in CRH gene expression in the NAcc during withdrawal and this effect was not observed in males. In contrast to females, adult males displayed an increase in biochemical markers of stress during nicotine exposure but not withdrawal from this drug. The sex differences in adults do not appear to be confounded by metabolism, since cotinine values were the same in male and female rats. Regarding age-group differences, adolescent males displayed larger effects in some markers of stress during nicotine exposure and withdrawal. This may have been related to the high amounts of nicotine that the adolescents received in order to produce equivalent cotinine values as adults.

4.2 Female adults displayed higher stress responses during withdrawal than males

The major finding of this dissertation is that adult females displayed greater increases in anxiety-like behavior, corticosterone levels, and changes in CRH gene expression in the NAcc during nicotine withdrawal as compared to males. First, adult females spent more time on the closed arm of the elevated plus maze during nicotine withdrawal as compared to males. Consistent with this, adult females also spent more time in the corner areas of the open field during nicotine withdrawal relative to males. Second, adult females displayed significant increases in plasma corticosterone levels during nicotine withdrawal as compared to males.

Third, adult females displayed an increase in CRH mRNA expression in the NAcc during nicotine withdrawal that was higher than males. These findings suggest that adult females experience greater behavioral and biological indices of stress during nicotine withdrawal as compared to males. These patterns of sex differences in adults were not observed during nicotine exposure, suggesting that these effects were produced by nicotine withdrawal.

The present findings are consistent with recent studies demonstrating that female rodents display enhanced anxiety-like behavior during nicotine withdrawal as compared to males. For example, female adult mice display more anxiety-like behavior on the elevated plus maze during nicotine withdrawal as compared to males (Kota et al., 2007; 2008). Similarly, Caldarone et al. (2008) showed that female mice display more anxiety-like behavior on the elevated plus maze following chronic oral nicotine intake as compared to males. Two recent reports also showed that adult female rats display higher plasma corticosterone levels during nicotine withdrawal as compared to males (Gentile et al., 2011; Skwara et al., 2012). The data presented in this dissertation contributes novel information to the literature regarding molecular markers of stress in the NAcc that are higher in females as compared to males. Taken together with the present findings, there is strong evidence to suggest that females experience greater stress during nicotine withdrawal as compared to males.

4.3 The NAcc modulates sex differences produced by nicotine withdrawal

Following nicotine exposure and withdrawal, changes in CRH expression were examined in the hypothalamus, amygdala and NAcc of male and female rats. During withdrawal, the largest sex differences were observed in the NAcc. The finding that withdrawal produced changes in stress markers in the NAcc may not be surprising given that intra-NAcc administration of CRH produces anxiety-like behavior on the elevated plus maze (Chen et al.,

2012). A recent report also demonstrated that the NAcc is strongly activated following presentation of a stressful stimulus (Noh et al., 2012). Interestingly, the latter report also showed that the strongest activation of the NAcc occurred following restraint stress, but not after stress produced by cold-water submersion. This suggests that the NAcc is differentially responsive to various types of stressors. The present findings suggest that the NAcc is a region involved in stress produced by nicotine withdrawal. Consistent with this, intra-NAcc administration of a non-selective CRH receptor antagonist reversed the decreases in brain reward function produced by nicotine withdrawal (Marcinkiewcz et al., 2009).

Previous studies have identified sex-dependent changes in the NAcc during withdrawal from other drugs of abuse. For example, morphine withdrawal produced a decrease in mu-opioid receptors in the NAcc of female but not male mice (Diaz et al., 2006). Also, multiple withdrawal periods from ethanol produced an increase in proteins involved in vesicular packaging and exocytosis in the NAcc of female but not in male rats (Bell et al., 2006). Cocaine withdrawal also produced an increase in cellular trafficking of delta opioid receptors in the NAcc of female, but not male rats (Ambrose-Lanci et al., 2008). Following repeated injections of nicotine, dopamine transporters were increased in the NAcc of female but not male rats (Harrod et al., 2004). Taken together with the present findings, there is strong evidence to suggest that the NAcc is an important brain structure in the mechanisms that modulate sex differences produced by withdrawal.

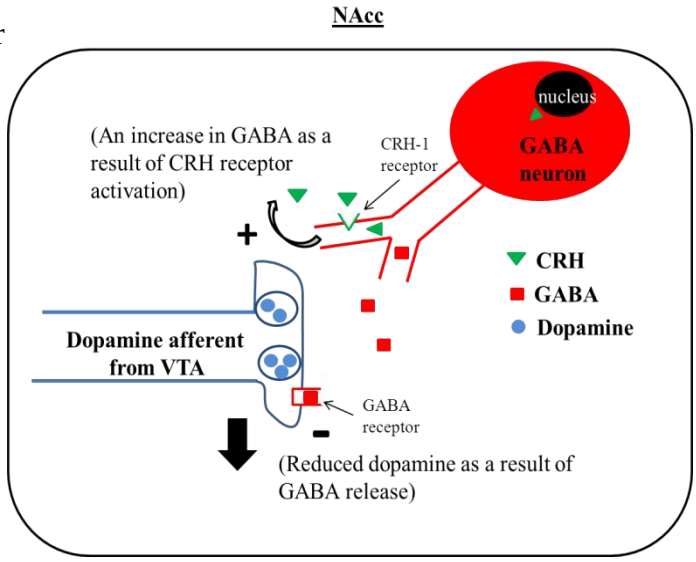
4.4 Putative mechanism of stress regulation of dopamine in the NAcc

Much work has demonstrated that a decrease in dopamine in the NAcc is a hallmark feature of withdrawal from drugs of abuse including alcohol, cocaine, and morphine (Broderick et al., 2004; Pothos et al., 1991; Rada et al., 2004; 2001; Weiss et al., 1992; Zhang et al., 2012).

Recent work in our laboratory has also demonstrated that synaptic levels of dopamine are reduced in the NAcc following withdrawal from nicotine (Natividad et al., 2010). A subsequent study demonstrated that the decreases in NAcc dopamine are modulated via amino acid regulation of dopamine systems. Specifically, during nicotine withdrawal decreases in NAcc dopamine were highly correlated with an increase in GABA levels in the cell body region of the mesolimbic pathway (Natividad et al., 2012). Based on this work, it is suggested that the decreases in dopamine produced by withdrawal are under direct inhibitory control from GABAergic systems in the mesolimbic pathway.

Much work has also shown that mesolimbic dopamine transmission is heavily modulated by the CRH stress system (Borgland et al., 2010; Chen et al., 2012; Izzo et al., 2005; Koob, 2010; Noh et al., 2012; Wanat et al., 2008). The anatomical location of CRH systems throughout terminal regions of the mesolimbic dopamine pathway suggests that stress systems modulate drug intake and relapse to drug-seeking behavior. In fact, several recent reviews have strongly encouraged research aimed at understanding the dysregulation of CRH systems as they contribute to the initiation, maintenance, and relapse of drug-seeking behavior (Borgland et al., 2010; George et al., 2012a; Koob et al., 2009; Koob & Kreek, 2007; Logrip et al., 2011). Stress systems appear to be particularly important with regard to tobacco use, since stress is a major negative affective consequence of nicotine withdrawal and is believed to drive relapse to smoking behavior (al'Absi, 2004; 2006; Bruijnzeel, 2012; Bruijnzeel & Gold, 2005). In support of this hypothesis, pre-clinical studies have shown that chronic nicotine self-administration sensitizes HPA axis activation in response to novel stressors (Chen et al., 2008; Yu et al., 2008; 2010).

The diagram to the right depicts our hypothesized mechanism for CRH modulation of NAcc dopamine during withdrawal. It is suggested that CRH systems modulate the decreases in NAcc dopamine via CRH receptors on inhibitory GABAergic interneurons. Specifically, it is suggested that CRH-1 receptor activation produces an increase in GABA levels that inhibit dopamine release in this region. The hypothesized mechanism was derived from studies examining the relationship between these systems in the NAcc as well as other brain regions. As stated previously, dopamine transmission in the NAcc is under direct inhibitory control from GABA interneurons that synapse onto dopamine terminals. This is evident by the observation that administration of a GABA agonist into the NAcc reduces local levels of dopamine in this region (Fu et al., 2012).



With regard to CRH in the NAcc, it is suggested that the effects of CRH are modulated within the local circuitry of this region. The presence of CRH (Swanson et al., 1983) and CRH mRNA (Koya et al., 2005) in the NAcc suggests that CRH may be synthesized locally. Support for this hypothesis is derived from studies showing that local CRH synthesis occurs within GABA interneurons of the hippocampus and locus coeruleus (Gallaghen et al., 2008; Ungless et al., 2010; Valentino et al., 1983). In addition, CRH receptors have also been identified in the NAcc (Aguilera et al., 2004; De Souza et al., 1985; Hsu et al., 2009). The CRH-1 receptors are hypothesized to regulate GABA inhibition of dopamine, as activation of CRH-1 receptors produces an increase in GABA transmission (Beckstead et al., 2009). The effects of CRH on

dopamine release appear to be dependent upon many factors. For example, a recent report showed that activation of CRH-1 receptors in the NAcc increased dopamine levels in this region (Chen et al., 2012). However, a latter report showed that following chronic stress, the regulation of CRH systems is switched such that activation of CRH receptors leads to a decrease in NAcc dopamine levels (Lemos et al., 2012). Based on the latter finding it is suggested that activation of CRH-1 receptors produces an increase in local GABA levels that leads to a decrease in dopamine during withdrawal. Our hypothesis regarding enhanced GABA transmission during withdrawal is supported by the finding that chronic CRH administration enhances GABA levels in the cortical terminals of the mesolimbic pathway (Kirby et al., 2008; Sirinathsinghji & Heavens, 1989). There is also support for the hypothesized mechanism from studies examining the relationship between these systems during withdrawal from other drugs of abuse. For example, withdrawal from alcohol has been shown to activate both GABA and CRH neurons in the prefrontal cortex of alcohol-dependent rats (George et al., 2012b). In the amygdala, withdrawal from alcohol has been associated with increases in CRH mRNA and GABA levels, an effect that is reversed by systemic administration of a CRH-1 receptor antagonist (Roberto et al., 2010). These studies provide evidence for a CRH-GABA interaction during withdrawal.

4.5 Sex differences in the proposed mechanism

There are several ways in which females may be more susceptible to stress during nicotine withdrawal. A recent review describes several potential avenues by which females are more sensitive to stimulation of CRH systems (Bangasser & Valentino, 2012). This review describes CRH as a substrate that orchestrates stress responses by activating the HPA axis and by acting as a neuromodulator in the brain. Their work has primarily focused on CRH modulation of norepinephrine systems in the locus coeruleus (LC), which coordinates arousal components of

the stress response. Hypersecretion of CRH in the LC is also a characteristic feature of many stress-related psychiatric disorders. Within the LC, female rats display higher levels of the CRH-1 receptor and heightened innervation by CRH as seen by immunostaining methods (Bangasser et al., 2012; Curtis et al., 2006). The CRH-1 receptor is a G-protein coupled receptor that transmits signals via intracellular second messenger systems (Aguilera et al., 2004; Dunn & Berridge, 1990; Kudielka & Kirschbaum, 2005). Regarding sex differences, Bangasser et al. (2010) found that the ratio of CRH-1 receptor to coupling of G-proteins was higher in female versus male rats. This suggests that the female CRH system has greater intracellular signaling capacity as compared to males.

The CRH receptor is internalized via the beta-arrestin2 protein. The beta-arrestin2 protein is an intracellular protein that internalizes the CRH-1 receptor into the cell cytoplasm and prevents it from being activated by CRH (Holmes et al., 2006). Using western blot techniques, it was demonstrated that female rats display lower levels of beta-arrestin2 than male rats (Bangasser et al., 2010). Based on this finding, it has been suggested that females are more responsive to CRH stimulation due to reduced internalization of the CRH-1 receptor as compared to males (Bangasser & Valentino, 2012). Thus, the possibility exists that the female CRH system may be more activated by CRH release during nicotine withdrawal as compared to males.

A final factor to consider is the influence of ovarian hormones on sex differences to stress produced by withdrawal. It is suggested that estrogen is a hormone that may enhance the effects of CRH systems in our proposed mechanism. This is based on studies showing that estrogen potentiates stress systems. For example, Viau et al. (2005) showed that CRH mRNA levels are higher in the hypothalamus of female versus male rats. Moreover, the increases in CRH mRNA

levels were higher in females with fully matured estrogen systems as compared to young adult females. These findings corroborate with studies comparing CRH levels across the 4-day estrous cycle in female adult rats. Specifically, the highest levels of CRH were observed during the proestrus phase, in which estrogen levels are highest as compared to other phases of the estrous cycle (Bohler et al., 1990; Nappi et al., 1997). Other work has also shown that direct activation of estrogen-beta receptors (ERβ) increase CRH mRNA expression *in vitro* (Chen et al., 2008; Lalmansingh & Uht, 2008; Zhu & Zhou, 2008). The estrogen gene sequence has also been shown to promote CRH gene transcription (Vamvakopoulos & Chrousos, 1993). Collectively, these studies suggest that estrogen enhances stress responses via facilitating CRH systems. Thus, estrogen may be an important factor that modulates sex differences produced by nicotine withdrawal.

4.6 Remaining questions to address in future studies

The studies in this dissertation have laid the groundwork for future studies examining the mechanisms of sex differences produced by nicotine withdrawal. The proposed framework suggests that decreases in NAcc dopamine levels produced by nicotine withdrawal are modulated via CRH systems that facilitate GABA inhibition in this region. During nicotine withdrawal, the possibility exists that female rats display a greater decrease in dopamine in the NAcc than males. The possibility also exists that this effect is due to greater GABAergic inhibition of dopamine in the NAcc. To our knowledge, these questions have not been examined and the role of ovarian hormones on the proposed mechanisms has not been studied.

The possibility also exists that brain regions other than the NAcc modulate sex differences produced by withdrawal. The present study examined changes in CRH gene expression in the hypothalamus and amygdala during nicotine exposure and withdrawal.

However, the finding that CRH mRNA was not altered in the hypothalamus may not be surprising, since CRH mRNA expression is not altered in the hypothalamus of male rats experiencing spontaneous nicotine withdrawal (Semba et al., 2004). Future studies are needed to examine whether the hypothalamus modulates other aspects that are sex-dependent during nicotine withdrawal.

The present study also found that adult males displayed a robust increase in CRH mRNA expression in the amygdala during nicotine exposure. A recent report showed that CRH levels were increased in the amygdala of male adult rats experiencing nicotine withdrawal (George et al., 2007). The rats in the latter study received a nicotine antagonist to precipitate withdrawal while nicotine was being delivered via an osmotic pump. The findings from this dissertation may be consistent with the George et al. study, since both reports show in increase in amygdala CRH systems during nicotine exposure. Thus, chronic nicotine exposure may produce an increase in CRH within the amygdala. In support of this hypothesis, high doses of nicotine produced an upregulation of CRH mRNA levels in the amygdala of male adolescent rats. Future studies are needed to determine the unique contribution of the amygdala to changes produces by nicotine exposure versus withdrawal from this drug. This is a challenge for future studies given that nicotine exposure is an inherent process by which withdrawal states are induced.

4.7 Limitations

One limitation of the present study is that our measures of gene expression do not directly reflect a change in CRH synthesis, receptor numbers, and/or release. Changes in gene expression do not directly reflect a change at the protein level. Thus, a limitation of this dissertation includes validation of functional proteins that reflect an actual change in CRH expression within the brain regions tested. Future studies could address these limitations by assessing CRH levels and CRH

protein levels using neurochemical and/or immunofluorescence techniques that directly assess functional changes at the protein level

Another consideration for the interpretation of these data is the order in which the behavioral tests were conducted. Animals were tested on the elevated plus maze first and then on the open field test. Since all rats were tested in this sequence, there may have been carry-over effects from one test to the other. Future studies might counter balance the order of these tests, such that half of the animals are tested first on the elevated plus maze and the other half is tested first on the open field.

Lastly, hormonal fluctuations across the 4-day estrous cycle may have influenced our results. However, we did not assess the phase in which the adult females were tested in. Future studies could address this limitation by testing different groups of adult rats during each phase of the 4-day estrous cycle. In addition, future studies may also employ ovariectomy procedures to examine whether ovarian hormones modulate stress responses produced by nicotine withdrawal.

4.8 Adolescents display less nicotine withdrawal than adults

Another major finding of this dissertation is that adolescent rats displayed reduced nicotine withdrawal as compared to adults. Specifically, both male and female adolescents displayed fewer somatic signs of withdrawal and behavioral markers of stress during withdrawal as compared to adult rats. These findings are consistent with much work from our laboratory demonstrating that adolescent rats display diminished nicotine withdrawal as compared to their adult counterparts (for a review, see O'Dell, 2009). For example, place aversion to an environment previously paired with nicotine withdrawal was lower in adolescent rats as compared to adults (O'Dell et al., 2007). Subsequent studies revealed that adolescent rats displayed reduced changes in dopamine and amino acid systems during nicotine withdrawal as

compared to adults (Natividad et al., 2010). Furthermore, nicotine-treated adolescents displayed reduced sensitivity to place aversion and decreases in NAcc dopamine levels produced by kappa opioid receptor activation versus adults (Tejeda et al., 2012). These findings are consistent with other laboratories showing that the behavioral effects of nicotine withdrawal are lower in adolescent rodents as compared to adults (Kota et al., 2007; 2008; Shram et al., 2008; Smith et al., 2006; Wilmouth & Spear, 2006). This dissertation extends this work by showing that adolescent females are also less sensitive to nicotine withdrawal as compared to adult females.

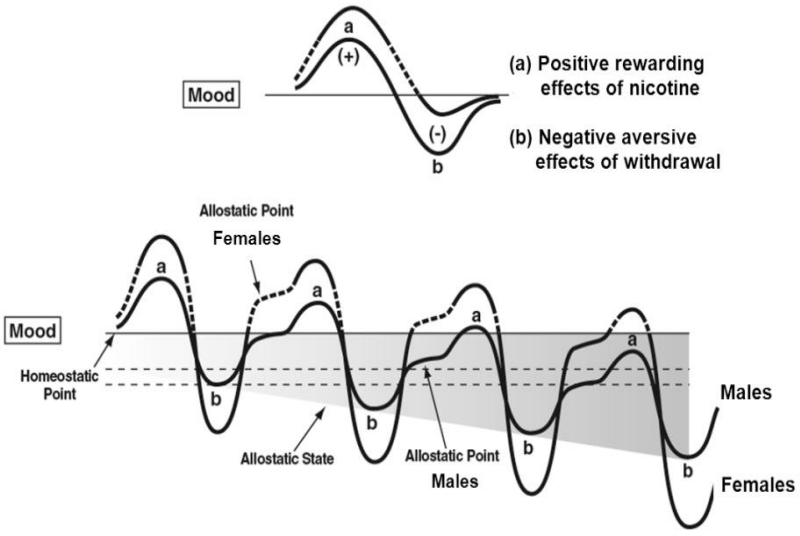
An important caveat is that adolescent males displayed an increase in anxiety-like behavior on the elevated-plus maze 24 hours after the removal of the nicotine pump. These rats also displayed a robust increase in CRH mRNA in the brain, suggesting that the adolescent males were stressed. A plausible explanation for these effects is that a high dose of nicotine was needed in order to produce equivalent cotinine levels as adults. Thus, the adolescent results may be related to strong carry-over effects of stress that persist 24 hours after the removal of the nicotine pump. The lack of stress effects in female adolescents was likely related to high tolerance to the aversive effects of nicotine, an effect that has been previously demonstrated (Torres et al., 2008). Thus, it is possible that adolescent males displayed stress-like responses due to exposure to high doses of nicotine. This presents a unique challenge for studies comparing age-group differences to stress produced by nicotine withdrawal, since high doses of nicotine are needed to compare to adults, and high doses of nicotine likely produces stress in adolescents.

4.9 Clinical implications

The opponent process theory of addiction posits that drug-seeking behavior is motivated by negative emotional states that result from counter adaptive processes that are unopposed in the absence of drug (Koob & Le Moal, 2001). This theory describes addiction

as a cycle that involves changes in neural systems that modulate positive rewarding effects (a-process) and negative states (b-process) produced by drug use (see figure below). Over time, the body has to vary all of the parameters to maintain stability in order to defend an altered set point produced by addiction (Koob & Le Moal, 2001). This allostatic state reflects a dysregulation in the mechanisms that mediate the relationship between the positive a-process that decreases over time and the negative b-process that becomes larger over time. As a result of an emerging negative b-process, the initial hedonic effects of drugs of abuse are believed to be masked and do not motivate drug seeking. Thus, negative affective states that increase with chronic drug use lead to drug use and promote relapse during abstinence.

The illustration to the right depicts our hypothesis regarding enhanced vulnerability to tobacco use in females (dotted line) compared to males (solid line). Our hypothesis is superimposed onto Koob and Le Moals' (2001) opponent process theory of addiction that only included one sold line.



The opponent process theory of addiction provides a useful framework for explaining the enhanced vulnerability to tobacco use among females. Overall, it is suggested that females display a stronger a and b process that together contribute to greater vulnerability to tobacco use in females as compared to males. With regard to positive effects, pre-clinical

studies have shown that the rewarding effects of nicotine are enhanced in female versus male rats. For example, acquisition of nicotine self-administration is faster in female versus male rats (Donny et al., 2000). Furthermore, on demanding schedules of reinforcement female rats display higher levels of operant responses for nicotine as compared to males (Chaudhri et al., 2005). Female mice also display higher consumption of a nicotine solution as compared to males in a two-bottle choice procedure (Klein et al., 2004). Work from our laboratory showed that female adolescent and adult rats display stronger preference for an environment paired with nicotine as compared to their male counterparts (Torres et al., 2008; 2009). The latter studies also showed that females were less sensitive to the aversive effects of nicotine. Reduced negative effects may contribute to the initiation of tobacco use since aversive effects of nicotine may limit tobacco use. Taken together, it is suggested that strong rewarding effects (a-process) of nicotine may contribute to enhanced vulnerability to tobacco use in females.

With regard to negative effects produced by withdrawal, it is suggested that females display a stronger b-process during abstinence than males. This is based largely on the present finding that nicotine withdrawal produced stronger behavioral and biological indices of stress in female versus male rats. This is consistent with pre-clinical studies from other laboratories showing that females display greater effects of nicotine withdrawal as compared to males (Caldarone et al., 2008; Gentile et al., 2011; Hamilton et al., 2010; Kota et al., 2007; 2008; Skwara et al., 2012). There is support for our hypothesis from clinical studies. Specifically, women smokers display more negative mood states, such as anxiety during abstinence than men (Perkins & Scott, 2008; Schnoll et al., 2007; Xu et al., 2008). Women also display elevated levels of cortisol relative to men during smoking abstinence (Hogle &

Curtin, 2006). Importantly, females also report that the anxiety-reducing effects of cigarettes are the main reason for smoking as compared to men (Perkins et al., 2009; 2012; Piper et al., 2010). Thus, these findings suggest that the strong aversive effects of withdrawal (b-process) also contribute to enhanced vulnerability to tobacco use in females.

Taken together, it is suggested that as compared to males, females experience stronger rewarding effects of nicotine and greater negative effects during withdrawal from this drug. Given that both rewarding effects and withdrawal processes contribute to tobacco use, we suggest that stronger effects in both domains contribute to greater vulnerability to tobacco use in females. Although social factors may also influence smoking susceptibility in women, the present study supports a biological mechanism involving greater stress produced by withdrawal in females. Specifically, these findings suggest that one plausible mechanism by which females are more vulnerable to tobacco use is intense stress produced by withdrawal. These observations suggest that female smokers may require specialized medications that alleviate intense stress produced by nicotine withdrawal. This dissertation work is an important first step toward developing novel therapeutic agents that may be more useful in smoking cessation in women. Given that most smoking cessation medications focus on alleviating withdrawal, one approach might include CRH antagonists in combination with other treatments, such as NRT or partial nicotine agonists. In fact, a recent review suggests that CRH receptor antagonists may be useful agents for alleviating negative mood states produced by drug withdrawal (Logrip et al., 2011). Future studies are needed to understand the complex interactions in the brain that modulate sex differences to tobacco use. Ultimately, this work is important towards reducing health disparities related to enhanced vulnerability to tobacco use among women.

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Figure 1

Blood plasma cotinine levels

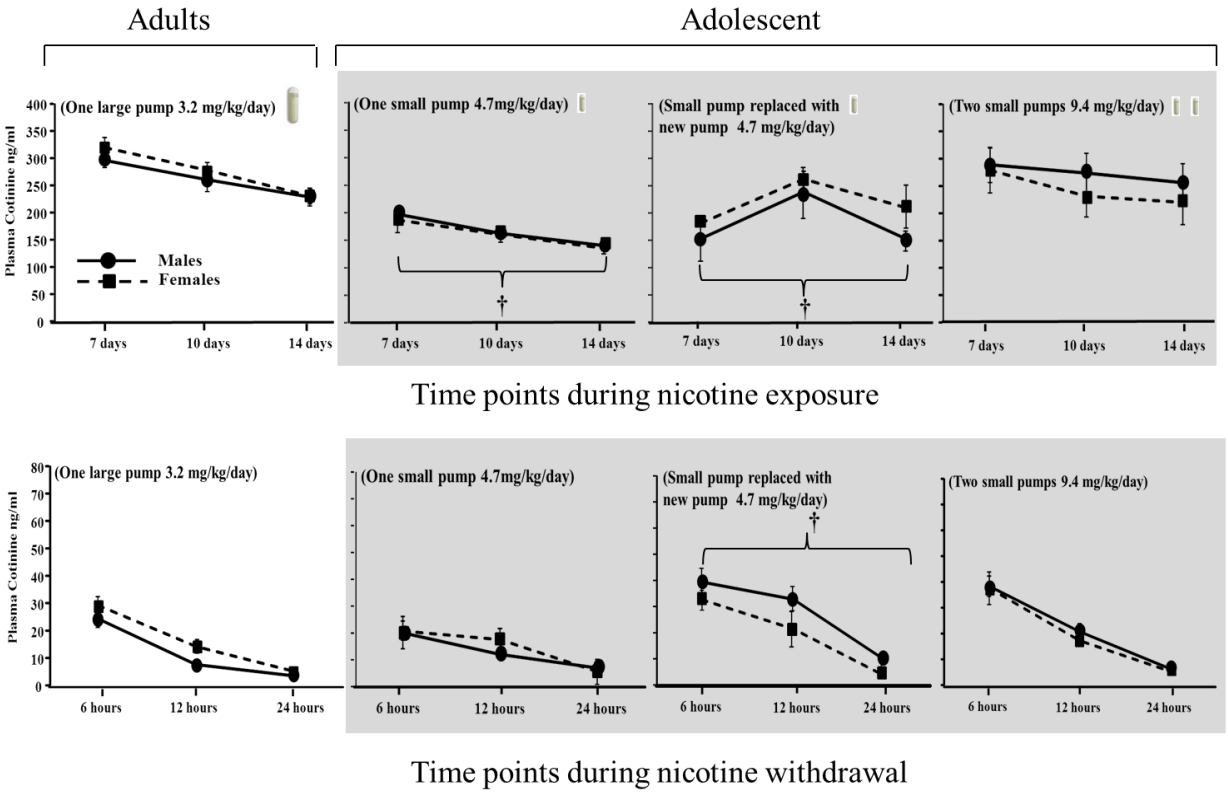

Figure 1 legend: This figure illustrates blood plasma cotinine levels (ng/ml±SEM) 7, 10, and 14 days during nicotine exposure (top row) and then 6, 12, and 24 hours after pump removal (bottom row) in adult and adolescent male and female rats. Adult rats (n=26) received a large pump (model 2ml2) that delivered nicotine 3.2 mg/kg for 14 days. Three separate groups of adolescent rats received a smaller model of pump (model 2002) that delivered: 1) a dose of 4.7 mg/kg/day for 14 days (n=17), 2) a dose of 4.7 mg/kg/day that was replaced after 7 days with a new pump that also delivered 4.7 mg/kg/day (n=17), and 3) a dose of 9.4 mg/kg/day that was evenly distributed in two small pumps (n=32). The dagger (†) denotes a significant difference across all time points relative to adults ($p \leq 0.05$).

Figure 2

Physical signs of withdrawal

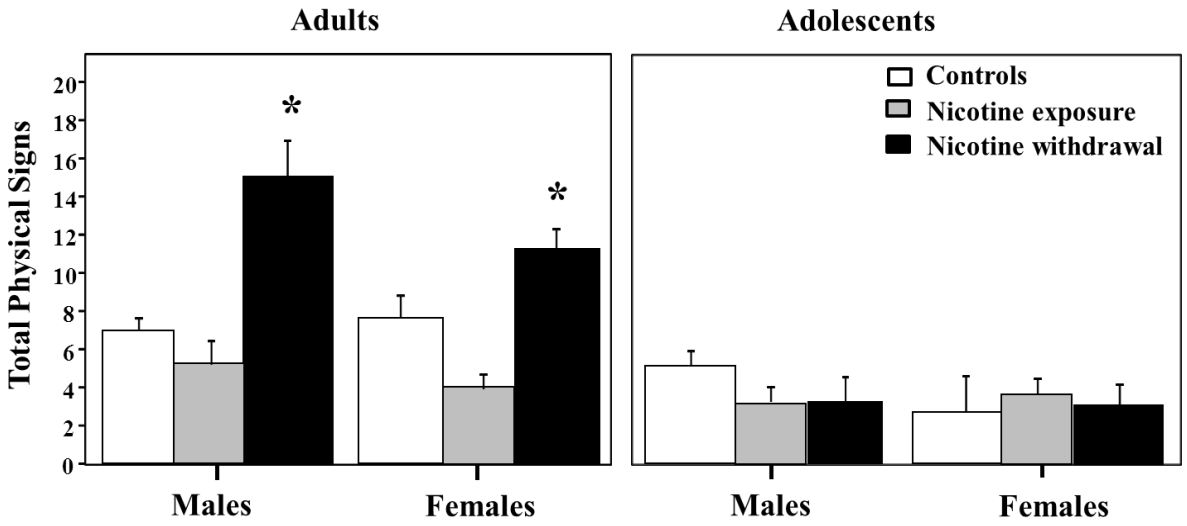

Figure 2 legend: This graph reflects total somatic signs of withdrawal (±SEM) during nicotine exposure and withdrawal in adult male (control n=13; nicotine exposure n=15; nicotine withdrawal n=10), adult female (control n=10; nicotine exposure n=15; nicotine withdrawal n=13), adolescent male (control n=6; nicotine exposure n=5; nicotine withdrawal n=5), and adolescent female (control n=5; nicotine exposure n=6; nicotine withdrawal n=5) rats. The asterisks (*) denote a significant difference from respective controls ($p \leq 0.05$).

Figure 3

Anxiety-like behavior on the elevated plus maze

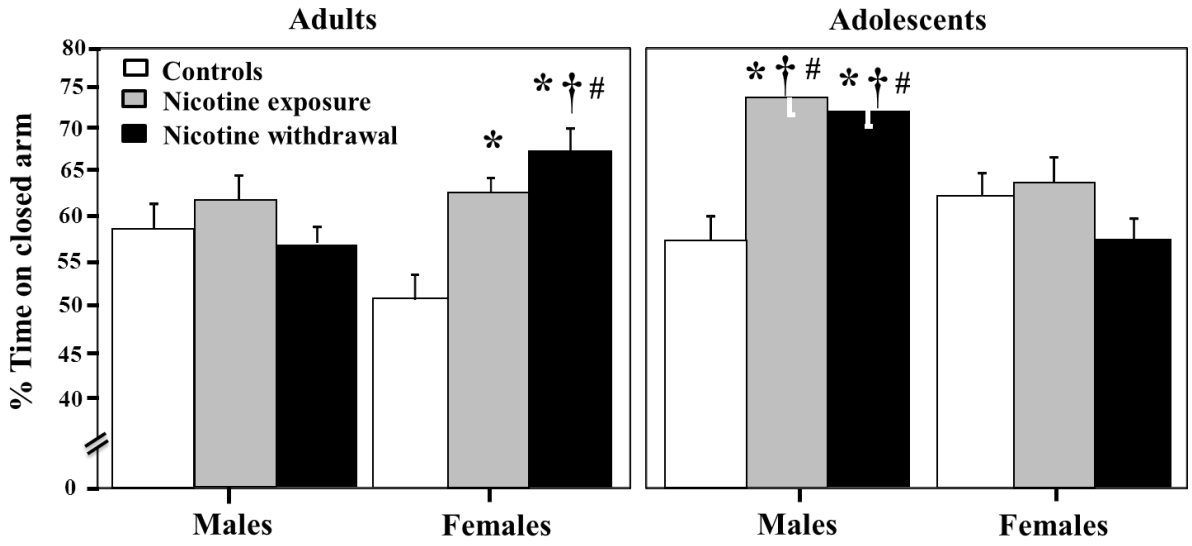

Figure 3 legend: This graph reflects percent time spent in the closed arm of the elevated plus maze during nicotine exposure and withdrawal in adult male (control n=13; nicotine exposure n=15; nicotine withdrawal n=9), adult female (control n=10; nicotine exposure n=16; nicotine withdrawal n=13), adolescent male (control n=6; nicotine exposure n=5; nicotine withdrawal n=5), and adolescent female (control n=6; nicotine exposure n=5; nicotine withdrawal n=5) rats. The asterisks (*) denote a significant difference from respective controls, the daggers (†) denote a significant difference between males and females, and the number signs (#) denote a significant difference between adults and adolescents ($p \leq 0.05$).

Figure 4

Anxiety-like behavior on the open field test

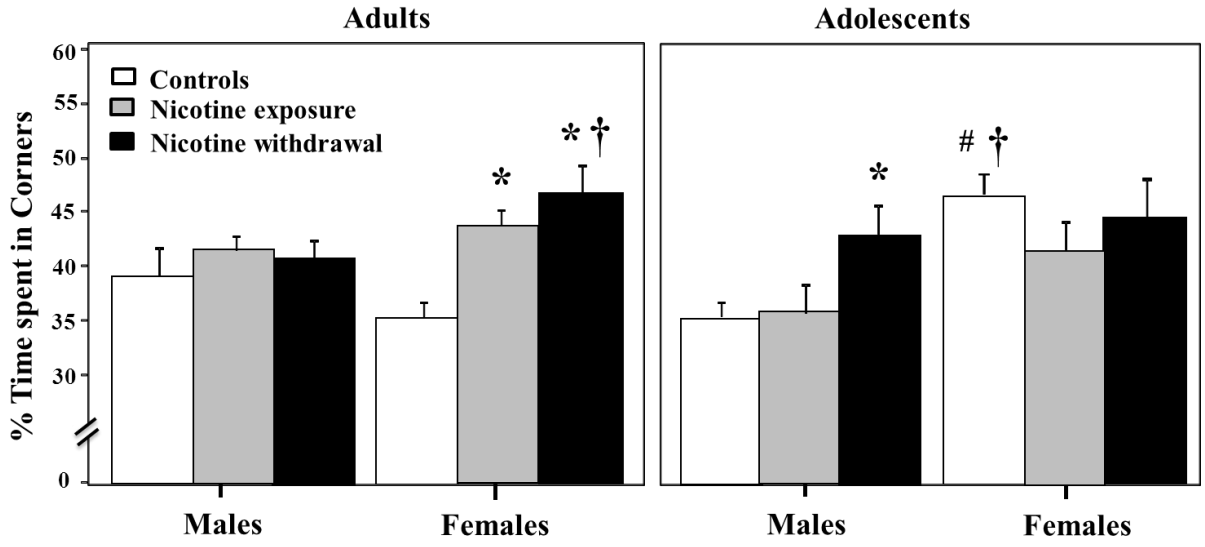

Figure 4 legend: This graph reflects percent time spent in the corner areas in the open field testing during nicotine exposure and withdrawal in adult male (control n=9; nicotine exposure n=15; nicotine withdrawal n=10), adult female (control n=10; nicotine exposure n=16; nicotine withdrawal n=13), adolescent male (control n=6; nicotine exposure n=5; nicotine withdrawal n=5), and adolescent female (control n=5; nicotine exposure n=5; nicotine withdrawal n=5) rats. The asterisks (*) denote a significant difference from respective controls, the daggers (†) denote a significant difference between males and females and the number sign (#) denotes a significant difference between adults and adolescents ($p \leq 0.05$).

Figure 5

Plasma corticosterone levels

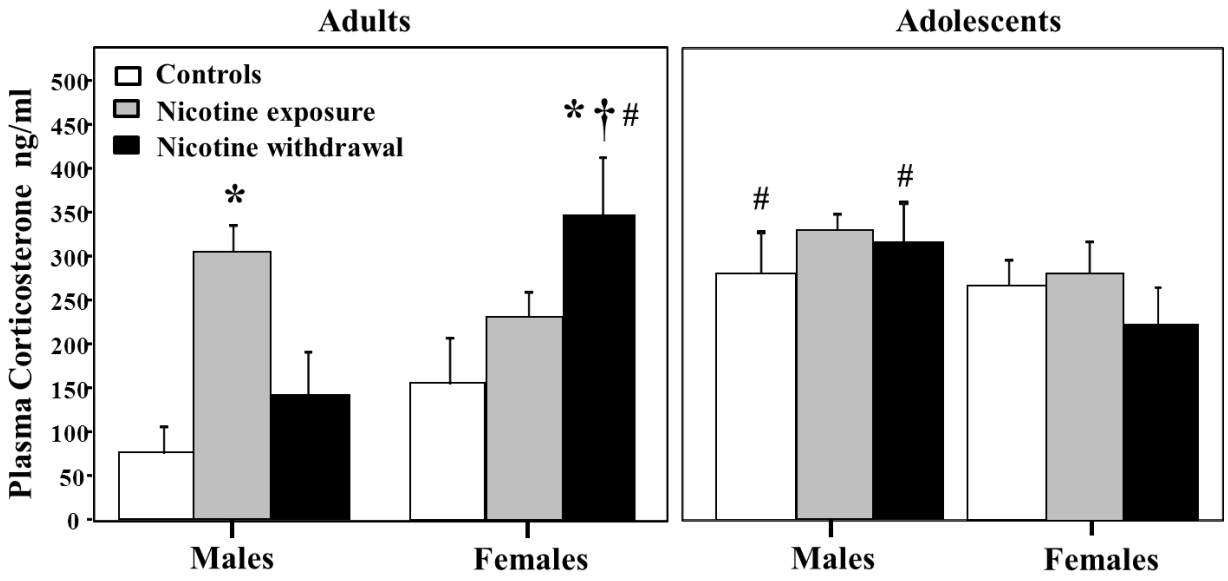

Figure 5 legend: This graph reflects total plasma corticosterone levels during nicotine exposure and withdrawal in adult male (control n=7; nicotine exposure n=9; nicotine withdrawal n=6), adult female (control n=8; nicotine exposure n=8; nicotine withdrawal n=8), adolescent male (control n=6; nicotine exposure n=5; nicotine withdrawal n=5), and adolescent female (control n=6; nicotine exposure n=5; nicotine withdrawal n=5) rats. The asterisks (*) denote a significant difference from respective control group, the dagger (†) denotes a significant difference between males and females, and the number signs (#) denote a significant difference between adolescents and adults ($p \leq 0.05$).

Figure 6

CRH gene expression in the NAcc

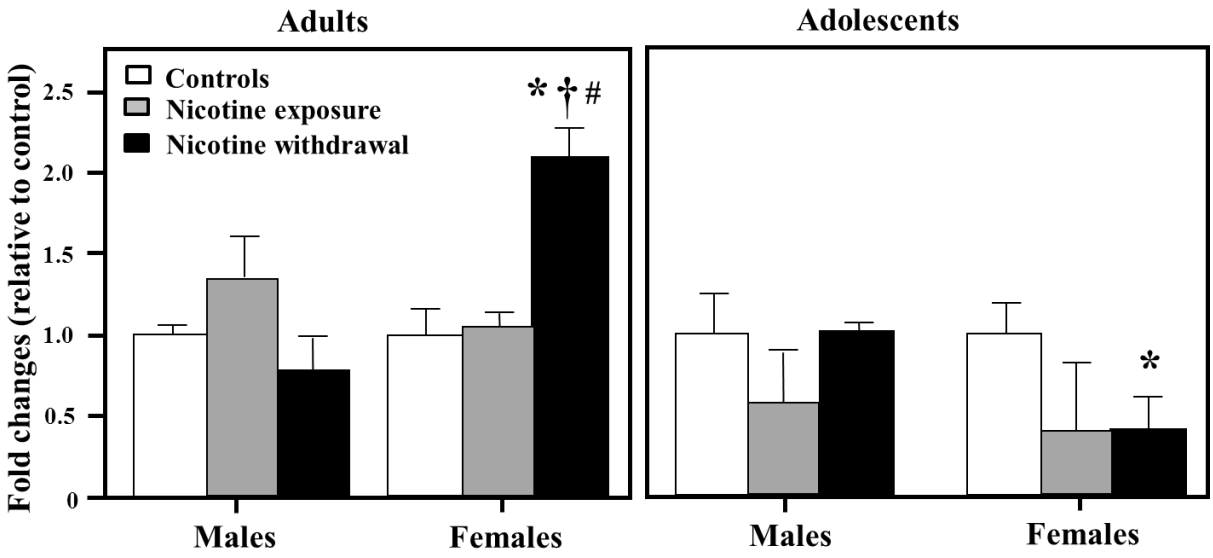

Figure 6 legend: This graph reflects CRH gene expression in the NAcc during nicotine exposure and withdrawal in adult male (control n=4; nicotine exposure n=4; nicotine withdrawal n=4), adult female (control n=4; nicotine exposure n=4; nicotine withdrawal n=4), adolescent male (control n=6; nicotine exposure n=5; nicotine withdrawal n=5), and adolescent female (control n=5; nicotine exposure n=4; nicotine withdrawal n=5) rats. The asterisks (*) denote a significant difference from respective controls, the dagger (†) denotes a significant difference between male and female rats, and the number sign (#) denotes a significant difference between adolescent and adult rats ($p \leq 0.05$).

Figure 7

CRH gene expression in the amygdala

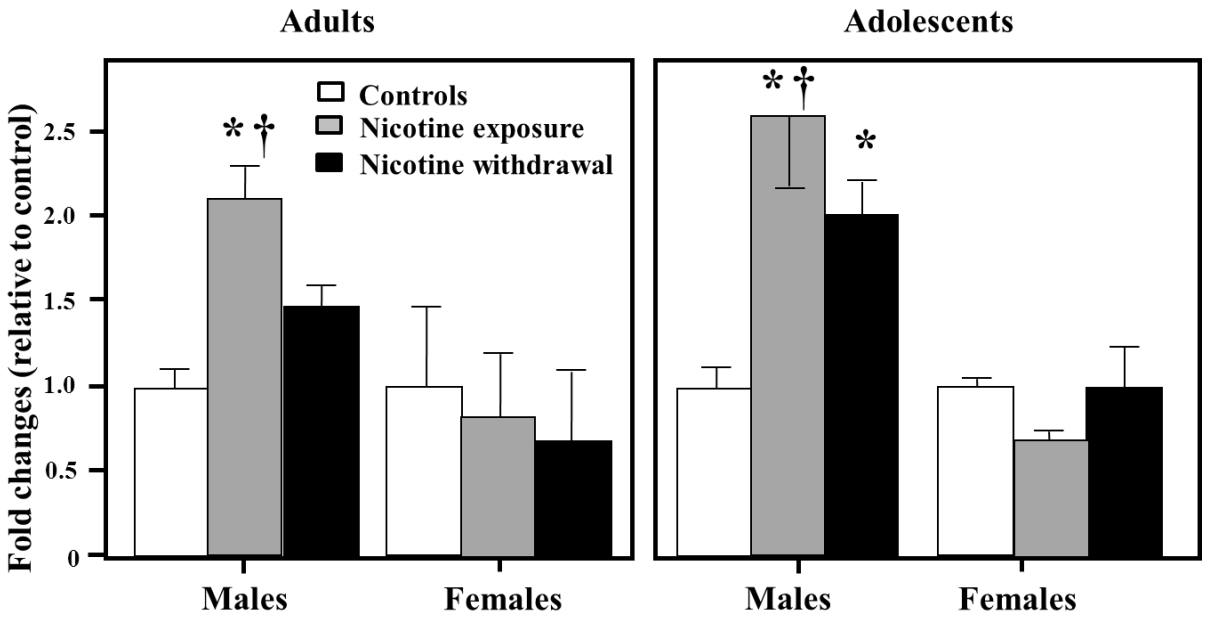

Figure 7 legend: This graph reflects CRH gene expression in the amygdala during nicotine exposure and withdrawal in adult male (control n=10; nicotine exposure n=5; nicotine withdrawal n=5), adult female (control n=7; nicotine exposure n=7; nicotine withdrawal n=4), adolescent male (control n=4; nicotine exposure n=6; nicotine withdrawal n=4), and adolescent female (control n=4; nicotine exposure n=4; nicotine withdrawal n=4) rats. The asterisks (*) denote a significant difference from respective controls ($p \leq 0.05$).

Figure 8

CRH gene expression in the hypothalamus

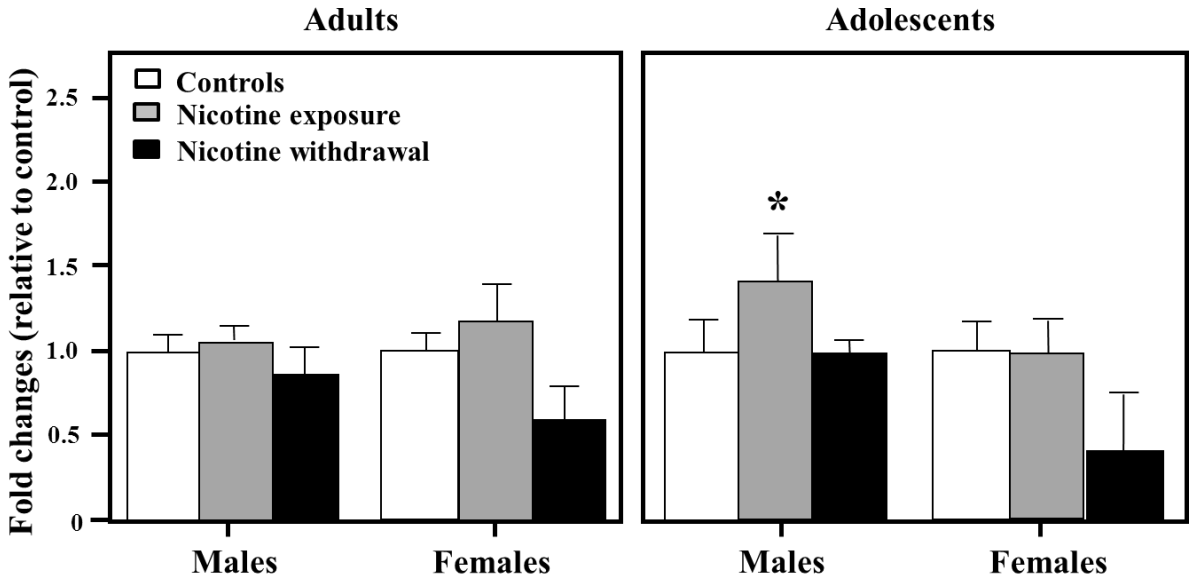

Figure 8 legend: This graph reflects CRH gene expression in the hypothalamus during nicotine exposure and withdrawal in adult male (control n=13; nicotine exposure n=13; nicotine withdrawal n=6), adult female (control n=6; nicotine exposure n=6; nicotine withdrawal n=4), adolescent male (control n=8; nicotine exposure n=5; nicotine withdrawal n=5), and adolescent female (control n=5; nicotine exposure n=5; nicotine withdrawal n=6) rats. The asterisks (*) denote a significant difference from respective controls ($p \leq 0.05$).

Curriculum Vitae

Oscar Valentin Torres was born to Luis Alfredo Torres and Maria Alma Torres in El Paso, Texas on February 14th, 1982. He graduated from W.H. Burges High School in the Spring semester of 2000 and entered the University of Texas at El Paso (UTEP) in the Fall semester of that academic year. Thereafter, he became involved in psychology research and completed an undergraduate honors thesis at the UTEP Department of Psychology. Oscar received his Bachelor of Science in the Fall semester of 2005 with departmental honors. In the Summer semester of 2006, Oscar became interested in neuroscience research and received a training fellowship from the National Science Foundation to work in the laboratory of Dr. Laura O'Dell. Her laboratory combines behavioral, biochemical and molecular techniques to study the mechanisms that mediate nicotine addiction vulnerable populations, such as adolescents and females. Oscar received his Master's degree in Experimental Psychology during the Spring semester of 2007. He then entered the Social, Cognition, and Neuroscience doctoral program at UTEP, where he continued his training and research with Dr. O'Dell. Oscar published two first-author papers and is co-author on four publications examining the role of nicotine reward and withdrawal in male and female rats. During his graduate career he presented 35 poster abstracts and seven oral presentations at scientific conferences. While pursuing his degree, Oscar was also a primary instructor for four courses including Introduction to Psychology, Behavior Modification, Statistical Methods, and Psychobiology at the UTEP. His dissertation work was supported by a dissertation fellowship from Dodson Dissertation Fellowship Program.

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Peer reviewed publications
1. Natividad, L.A , Parsons, L. A., Torres, O.V., O'Dell, L.E . (2012). Adolescent rats are resistant to adaptations in excitatory and inhibitory mechanisms that modulate mesolimbic dopamine during nicotine withdrawal. Journal of Neurochemistry, in press.

2. Tejeda, H.A., Natividad, L.A., Orfila, J. E., Torres, O.V., O'Dell, L.E. (2012). Dysregulation of kappa-opioid receptor systems by chronic nicotine modulate the nicotine withdrawal syndrome in an age-dependent manner. Psychopharmacology, in press.

3. Natividad, L.A., Tejeda, H.A., Torres, O.V., O'Dell, L.E. (2010). Nicotine withdrawal produces a decrease in extracellular levels of dopamine in the nucleus accumbens that is lower in adolescent versus adult male rats. Synapse, 64(2),136-145.

4. Torres, O.V., Natividad, L.A., Tejeda, H.A., Van Weelden, SA., O'Dell, L.E. (2009). The rewarding and aversive effects of nicotine in female rats are age-, hormone-, and sex-dependent. Psychopharmacology, 206(2), 303-12.

5. Torres, O.V., Tejeda, H.A., Natividad, L.A., O'Dell, L.E. (2008). Enhanced vulnerability to the rewarding effects of nicotine during the adolescent period of development. Pharmacology, Biochemistry and Behavior, 90, 658-663.

6. O'Dell, L.E., Torres, O.V., Natividad, L.A., Tejeda, H.A. (2007). Adolescent nicotine exposure produces less affective measures of withdrawal relative to adult nicotine exposure in male rats. Neurotoxicology and Teratology, 29(1), 17-22.

Publications currently under review

1.Torres, O.V., Walker, E. M., Blanca, S. B., O'Dell, L.E. Female rats display enhanced rewarding effects of ethanol that are hormone dependent.

Awards and Funding

1. January, 2012: Received the Dodson Doctoral Fellowship from the University of Texas at El Paso.

2. August, 2011: Received a travel award via the National Hispanic Science Network (NHSN) to attend the NHSN international conference in Miami, FL.

3. June, 2011: Primm-Singleton Underrepresented Population award to attend the College on Problems of Drug Dependence, Hollywood, FL.

4. March, 2011: Received travel funds to attend the Biology, Behavior, and Chemistry conference in San Antonio, TX.

5. June, 2010: Received a summer internship to attend the Marine Biology laboratories in Woodshole, MA and attend the Specialized Program in Neuroscience Ethics and Survival (SPINES) course.

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9. October, 2008: Received a travel award via the National Hispanic Science Network (NHSN) to attend the NHSN international conference in Washington, DC.

10. January, 2008: Received a Graduate Excellence Scholarship Award from the Department of Psychology at the University of Texas at El Paso to cover tuition costs.

11. January, 2005: Received a training fellowship from the National Science Foundation (Support of Mentors and Students from Under Represented Minorities) program to participate in neuroscience research during the summer in the laboratory of Dr. Laura E. O'Dell.

12. August, 2000: Received an Undergraduate University Academic Scholarship from the University of Texas at El Paso.

Research Training

June 2010- August 2010 Marine Biological Laboratories (MBL)
Title: Summer doctorate student trainee
Duties: I gained experimental and technical knowledge with electrophysiology. I conducted research at Marine Biological Laboratories, Woods Hole, MA while taking a course entitled Specialized Programs in Neuroscience, Ethics and Survival (SPINES). I gained experience in basic theory and methods in electrophysiology.
Experience: I conducted research using In-vitro extracellular recordings, and gained knowledge with ethical issues in neuroscience research.
Contact: Joe L Martinez, Ph.D. University of Texas at San Antonio, Department of Biology
San Antonio, TX 18249 Phone: (210) 458-4279 E-mail: JMartinez@utsa.edu

May 2009-August 2009: National Institute on Drug Abuse (NIDA)
Title: Summer doctorate student trainee
Duties: I gained research experience in molecular techniques to examine the effects of methamphetamine pre-conditioning and toxicity in rodent models. I conducted research in an area other than academia and became acquainted with research in a government setting.
Experience: I learned RNA extraction and synthesis of cDNA using neuronal tissue. I also conducted research using real-time polymerase chain reaction (RT-PCR) techniques and analysis of microarrays. Contact: Jean Cadet, M.D. National Institute on Drug Abuse, Molecular Neuropsychiatry Division Baltimore, MD 21224 Phone: (443) 740-2656 E-mail: Jcadet@intra.nida.nih.gov

May 2008-August 2008: The University of Texas at El Paso, Department of Biology
Title: Summer doctorate student trainee.
Duties: Participate and design molecular neuroscience experiments examining the mechanisms that mediate methamphetamine toxicity in rodent brain tissue and cell culture.
Experience: I learned brain extraction and isolation of neuronal structures from rodents. I also gained research experience using biochemical techniques such as western blot and ELISA preparations. Contact: Manuel Miranda, Ph.D. University of Texas at El Paso, Department of Biology El Paso TX, 79968 Phone: (915) 747-6645 E-mail: mmiranda3@utep.edu

August 2007-Current: The University of Texas at El Paso, Department of Psychology
Title: Ph.D. student in the Social, Cognition, and Neuroscience program.
Duties: Conduct and design behavioral neuroscience experiments examining the mechanisms that mediate developmental and sex differences to nicotine reward and withdrawal.
Experience: I gained research experience in behavioral testing such as conditioned place procedures, IVSA procedures, elevated plus maze and open field test procedures, qRT-PCR and ELISAs. I also gained knowledge in rodent surgical procedures. In addition, I served as the

primary breeder for the rat vivarium. Contact: Laura E. O'Dell, Ph.D. University of Texas at El Paso, Department of Psychology El Paso TX, 79968 Phone: (915) 747-6557 E-mail: lodell@utep.edu

Teaching Experience

August 2012- December 2012: The University of Texas at El Paso
Title: Instructor for the Psychology Department at UTEP
Duties: I served as the primary instructor for Psychobiology. My duties include giving lectures, leading class discussions and grading exams.
Contact: Stephen L. Crites, Ph.D. University of Texas at El Paso, Department of Psychology
El Paso TX, 79968 Phone: (915) 747-6571 E-mail: scrites@utep.edu

January 2012- May 2012: The University of Texas at El Paso
Title: Instructor for the Psychology Department at UTEP
Duties: I served as the primary instructor for Behavior Modification. My duties consisted of preparing and directing lectures as well as leading class discussions and grading exams.
Contact: Theodore V. Cooper, Ph.D. University of Texas at El Paso, Department of Psychology
El Paso TX, 79968 Phone: (915) 747-6551 E-mail: tvcooper@utep.edu

August 2011- December 2011: The University of Texas at El Paso
Title: Instructor for the Psychology Department at UTEP
Duties: I served as the primary instructor for Statistical Methods in Psychology. My duties consisted of preparing and directing lectures, grading exams, homeworks and tutoring students during office hours.
Contact: Stephen L. Crites, Ph.D. University of Texas at El Paso, Department of Psychology
El Paso TX, 79968 Phone: (915) 747-6571 E-mail: scrites@utep.edu

January 2010- May 2010: The University of Texas at El Paso
Title: Instructor for the Psychology Department at UTEP
Duties: I served as the primary instructor for General Experimental laboratory in Psychology. My duties consisted of preparing and directing lectures as well as leading class discussions, grading exams, papers and tutoring students during regular office hours.
Contact: Stephen L. Crites, Ph.D. University of Texas at El Paso, Department of Psychology
El Paso TX, 79968 Phone: (915) 747-6571 E-mail: scrites@utep.edu

January 2009-August 2009: The University of Texas at El Paso
Title: Instructor for the Psychology Department at UTEP
Duties: I served as the primary instructor for Introduction to Psychology s. My duties consisted of preparing and directing lectures as well as leading class discussions, grading exams, papers and tutoring students during regular office hours.
Contact: Edward Castañeda, Ph.D. University of Texas at El Paso, Department of Psychology
El Paso TX, 79968 Phone: (915) 747-6558 E-mail: ecastaneda9@utep.edu

January, 2005-May, 2007: The University of Texas at El Paso.
Title: Teaching assistant.

Duties: I worked as a teaching assistant for the Psychology Department at UTEP. I was responsible for proctoring exams, organizing, maintaining class grades and assisting undergraduate students in their understanding of the material. Courses: Statistics, Psychobiology, Drugs and Behavior
Contact: Laura E. O'Dell, Ph.D. University of Texas at El Paso, Department of Psychology
El Paso TX, 79968 Phone: (915) 747-6557 E-mail: lodell@utep.edu

Oral Presentations

1. (2011) College on Problems of Drug Dependence (CPDD); Hollywood, FL.

2. (2011) Behavior, Biology and Chemistry; San Antonio, TX.

3. (2010) Special Program in Neuroscience, Ethics and Survival (SPINES), Marine Biological Laboratories (MBL); Woods Hole, MA.

4. (2010) Behavior, Biology and Chemistry: Translational Research in Addiction Meeting; San Antonio, TX.

5. (2009) National Hispanic Science Network (NHSN) on Drug Abuse; Miami, FL.

6. (2009) Behavior, Biology and Chemistry: Translational Research in Addiction Meeting; San Antonio, TX

7. (2007) University of Texas at El Paso, Seminar Lecture Series Presentation; El Paso, TX

Poster Presentations

1. Natividad, L.A., Orfila, J.E., Torres, O.V., Parsons, L.H., O'Dell, L.E. Adolescent rats are resistant to adaptations in excitatory and inhibitory mechanisms that modulate mesolimbic dopamine during nicotine withdrawal. National Hispanic Science Network (NHSN) meeting, 2011.

2. Natividad, L.A., Parsons, L.H., Orfila, J.E., Torres, O.V., O'Dell, L.E. Periadolescent rats are resistant to adaptations in excitatory and inhibitory mechanisms that modulate mesolimbic dopamine during nicotine withdrawal. Society for Neuroscience (SFN), 2011.

3. Natividad, L.A., Escalante, E., Mangubat, M., Chang-Sung., S., Torres., O.V., Friedman, T.C., and O'Dell, L.E., Age differences in food-intake and the weight-suppressant effects of self-administered nicotine. Endocrine Society, 2011.

4. Natividad, L.A., Orfila, J.E., Torres, O.V., Parsons, L.H., O'Dell, L.E. Developmental differences in nicotine withdrawal are mediated via enhanced excitatory and reduced inhibitory mechanisms that regulate dopamine transmission in the mesolimbic pathway. Behavior, Biology and Chemistry (BBC), 2011.

5. Walker, E. M., Beas, B.S., Muñiz, A.K., Torres, O.V. and O'Dell, L. E. The rewarding effects of alcohol are dose-dependently enhanced in female versus male rats. Behavior, Biology and Chemistry, 2011.

6. Torres, O.V., Natividad, L.A., Walker, E.M., Muñiz, A.K., and O'Dell, L.E . Nicotine withdrawal enhances anxiety-like behavior in female versus male rats. Behavior, Biology and Chemistry, 2011.

7. Torres, O.V., Natividad, L.A., Byers, D.M., O'Dell, L.E. Developmental and sex differences in the expression of the molecular targets in a rat model of nicotine withdrawal. BBC, 2010.

8. Natividad, L.A., Escalante, E., Torres, O.V., Tejeda, H.A., Friedman, T.C., O'Dell, L.E. Age differences in the rewarding and weight-suppressant effects of nicotine. (SFN), 2010.

9. Torres, O.V., Natividad, L.A., Walker, E. M., Muniz, A., Byers, D. M., and O'Dell, L.E. Behavioral, biochemical, and molecular indices of nicotine withdrawal: Differential impact of sex on stress-related markers. Society for Neuroscience, 2010.

10. Natividad, L.A., Roman, F., Torres, O.V., Tejeda, H.A., and O'Dell, L.E. Exposure to nicotine during adolescence alters intake of the drug later in adulthood. National Hispanic Science Network on Drug Abuse, 2009.

11. Torres, O.V., Muniz, A., Roman, F., Beas, B.S., Natividad, L.A., and O¹Dell, L.E. Nicotine withdrawal is diminished during adolescence in female and male rats. NHSN, 2009.

12. Natividad, L.A., Tejeda, H.A., Torres, O.V., Castañeda E., and O'Dell, L.E. The neurochemical effects of nicotine withdrawal on dopamine transmission in the nucleus accumbens are lower in adolescent relative to adult rats. American Psychological Association, 2009.

13. Natividad, L.A., Roman, F., Tejeda, H.A., Torres, O.V., Castañeda E., and O'Dell, L.E. Diminished neurochemical effects of nicotine withdrawal in adolescent versus adult rats. Biology, Behavior, and Chemistry, 2009.

14. Torres, Oscar V., Natividad, L.A., Byers, Donna M., Tejeda, Hugo A. and O'Dell, Laura E. Nicotine withdrawal enhances anxiety-like behavior and expression of stress-related genes in female versus male rats. Biology, Behavior, and Chemistry, 2009.

15. Orfila J.E., Tejeda H.A., Natividad L.A., Torres, O.V., Castañeda E., and O'Dell L.E. The behavioral and neurochemical effects produced by kappa-opioid receptor stimulation are diminished in nicotine-dependent adolescent versus adult rats. Biology, Behavior, and Chemistry, 2009.

16. Natividad, L.A., Torres, O.V., Tejeda, H.A., Castañeda, E., and O'Dell, L.E. The neurochemical effects of nicotine withdrawal are different in adolescent and adult rats. National Hispanic Science Network, 2008.

17. Tejeda, H.A., Torres, O.V., Natividad, L.A., Orfila, J.R., Castañeda, E., and O'Dell, L.E. Stimulation of kappa opioid receptors elicits nicotine withdrawal in adult but not adolescent rats. NHSN, 2008.

18. Torres, O.V., Natividad, L.A., Tejeda, H.A., and O'Dell, L.E. The rewarding effects of nicotine are age-, hormone- and sex-dependent in rats. National Hispanic Science Network, 2008.

19. Natividad, L.A., Tejeda, H.A., Torres, O.V., Castañeda, E., and O'Dell, L.E. Robust developmental differences to the neurochemical effects of nicotine withdrawal are not observed following nicotine administration in adolescent versus adult rats. Society for Neuroscience, 2008.

20. Tejeda, H.A., Natividad, L.A., Torres, O.V., Castañeda, E., and O'Dell, L.E. The behavioral and neurochemical effects produced by kappa-opioid receptor stimulation are diminished in nicotine-dependent adolescent versus adult rats. Society for Neuroscience, 2008.

21. Torres, O.V., Van Weelden, S.A., Natividad, L.A., Tejeda, H.A., B.S., Beas, and O'Dell, L.E. The rewarding effects of nicotine are enhanced in female adolescent rats relative to adults that display rewarding or aversive effects in a hormone-dependent manner. Society for Neuroscience, 2008.

22. Natividad, L.A., Tejeda, H.A., Torres, O.V., and O'Dell, L.E. Diminished neurochemical effects of nicotine withdrawal in adolescent versus adult rats. College on Problems of Drug Dependence, 2008.

23. Tejeda, H.A., Torres, O.V., Natividad, L.A., Beas, B.S., and O'Dell, L.E. Stimulation of kappa-opioid receptors induces the behavioral effects of nicotine withdrawal in nicotine-dependent adult but not adolescent rats. Society for Research on Nicotine and Tobacco, 2008.

24. Byers, D.M., Natividad, L.A., Tejeda, H.A., Torres, O.V., and O'Dell, L.E. Developmental and sex differences in the expression of key molecular targets during nicotine withdrawal. Society for Research on Nicotine and Tobacco

25. Torres, O.V., Natividad, L.A., Tejeda, H.A., and O'Dell, L.E. The rewarding effects of nicotine are enhanced during adolescence in both male and female rats. Society for Research on Nicotine and Tobacco, 2008.

26. Natividad, L.A., Torres, O.V., Tejeda, H.A., and O'Dell, L.E. Pre-exposure to nicotine during adolescence facilitates nicotine self-administration in adult rats given intermittent access to escalating nicotine doses. Society for Neuroscience, 2007.

27. Tejeda, H.A., Natividad, L.A., Torres, O.V., and O'Dell, L.E. Stimulation of kappa-opioid receptors elicits nicotine withdrawal in adult but not adolescent rats. Society for Neuroscience, 2007.

28. Torres, O.V., Tejeda, H.A., Natividad, L.A., and O'Dell, L.E. The rewarding effects of nicotine are enhanced in female adolescent rats and in adult females in an estrous-dependent manner. Society for Neuroscience, 2007.

29. Byers, D.M., Natividad, L.A., Tejeda, H.A., Torres, O.V., and O'Dell, L.E., Characterization of gene targets of nicotine withdrawal in male and female adolescent and adult rats. Society for Neuroscience, 2007.

30. Torres, O.V., Tejeda, H.A., Natividad, L.A., and O'Dell, L.E. Reduced nicotine withdrawal may contribute to enhanced tobacco use during adolescence. National Hispanic Science Network on Drug Abuse Meeting, 2006.

31. Natividad, L.A., Torres, O.V., Tejeda, H.A., and O'Dell, L.E. Nicotine withdrawal produces a decrease in dopamine release in the nucleus accumbens of adult, but not adolescent rats. Society for Neuroscience, 2006.

32. Torres, O.V., Tejeda, H.A., Natividad, L.A., and O'Dell, L.E. Enhanced nicotine reward and diminished nicotine withdrawal in adolescent versus adult rats. Society for Neuroscience, 2006.

33. O'Dell, L.E., Natividad, L.A., Torres, O.V., and Tejeda, H.A. The affective properties of nicotine withdrawal are diminished in adolescent versus adult rats. College on Problems of Drug Dependence, 2005.

34. Torres, O.V., Natividad, L.A., Tejeda, H.A., and O'Dell, L.E. Diminished nicotine withdrawal in adolescent rats: Implications for vulnerability to addiction. Faculty for Undergraduate Neuroscience at the Society for Neuroscience Meeting, 2005.

35. Taylor, T. S., Torres, O.V., Hosch, M. H. The unknown voice: an examination of juror anonymity on verdicts. The American Psychology and Law society, 2005

Professional References

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