STORM, STRESS, AND NICOTINE: EXPLORING THE INTERACTIVE EFFECTS OF ADOLESCENT STRESS AND ADOLESCENT NICOTINE ON THE DEVELOPMENT OF LONG-TERM LEARNING DEFICITS

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by
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ABSTRACT

In order to continue the decline of smoking prevalence, it is imperative to understand the factors that contribute to the development of nicotine and tobacco addiction, such as adolescent initiation of nicotine use, stress, and the interaction of adolescent stress and adolescent nicotine. Both clinical and preclinical literature indicates that adolescent, but not adult, nicotine administration leads to long-lasting impairments in learning and memory and affect. Specifically, chronic nicotine treatment beginning in early adolescence or late adolescence resulted in adult deficits in contextual fear learning. However, the current study demonstrated that these adult learning deficits did not occur solely from nicotine administration. Rather, an interaction between adolescent nicotine and adolescent stress resulted in adult learning deficits in contextual fear. Additionally, it was found that dietary choline supplementation that began immediately following cessation of adolescent nicotine treatment and continued through a protracted abstinence period lasting thirty days reversed adult deficits in contextual fear. Finally, the current study found that adolescent nicotine exposure beginning at either early adolescence or late adolescence increased depressive-like behaviors, but not anxiety-like behaviors, following a protracted abstinence period. In contrast, chronic nicotine treatment in adult increased anxiety-like behaviors measured by the elevated plus-maze following a protracted abstinence period. The work encompassed in this dissertation suggests that the interactions between adolescent stress and adolescent nicotine increases the risk for developing cognitive and affective impairments, which may promote continued use of nicotine in adulthood.

I dedicate my dissertation to the lost souls I've known in my life who started with nicotine as angtsy teens and progressed to far worse.

I told you I would help you.

This is how I did it.

ACKNOWLEDGMENTS

First, I would like to acknowledge my core dissertation committee, Dr. Thomas Gould, Dr. Debbie Bangasser, and Dr. Jason Chein. Without their dedication to mentorship and scientific inquiry I would not feel half as confident in my future pursuits as a scientist. I would also like to acknowledge Dr. Deborah Drabick, Dr. Lisa Briand, and Dr. Vinay Parihk for reviewing my dissertation. To all of my committee members, I am truly grateful for never letting me settle for something that was just good enough and always encouraging me to be the best I could. A special acknowledgment goes to Dr. Thomas Gould who not only provided my foundation for being part of the next generation of science, but who also provided a listening ear through personal setbacks. Without his compassion and commitment to finding home-work balance I would not be nearing the completion of this degree.

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Finally. I must acknowledge my biggest supporter and cheerleader, my husband, Ryan Holliday. Whenever I could not believe in myself Ryan was always there to remind

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TABLE OF CONTENTS

Page

LIST OF FIGURES

Figure Page

LIST OF TABLES

CHAPTER 1

INTRODUCTION

Tobacco use is a leading cause of preventable death and despite the known risks associated with smoking 18.1% of Americans are every day smokers (CDC, 2014). According to the Center for Disease Control (CDC, 2014), 70% of current smokers want to quit and 40% of smokers have reported attempts to quit but have failed in the previous year (CDC, 2011). The prevalence of everyday smokers has leveled off at 20% of the population after dramatically declining over the last several decades. One reason for the steadfast prevalence rates may be due to the effects of adolescent nicotine abuse. Pre-clinical research in the last 10 years implicates adolescence as a critical period for enduring effects caused by nicotine exposure (Torres et al., 2008; Slotkin et al., 2008). Examining the effect of nicotine in adolescence is especially important because 90% of adult smokers initiate smoking prior to turning 20 (United States Department of Health and Human Services, 1994) and the younger the age of smoking initiation the more likely an individual will become dependent on tobacco in adulthood. Younger initiation of smoking is also associated with greater severity of nicotine dependence, which in turn decreases the likelihood of quitting (Chassin et al. 1990; Colby et al. 2000; Macy et al., 2012). In fact, the 2012 Surgeon General's report stated that if youth smoking prevention efforts had been sustained between 1997 and 2003, we could have nearly 3 million less young smokers today. Unfortunately, the CDC has recently reported that the use of e-cigarettes among adolescents nearly doubled from 2011 to 2012 and that 75% of those reporting e-cigarette use also smoked conventional cigarettes (CDC, 2014) indicating adolescent nicotine abuse remains a problem that needs to be addressed.

Adolescent nicotine use may lead to affective and cognitive disorders later in life. Several studies have observed a relationship between adolescent smoking and depressive symptoms later in life. For example, in a longitudinal study tracking adolescents and young adults over a period of 13 years, it was found that adolescent smoking was predicted of depressive symptoms in adulthood (Brook et al., 2004). Even more compelling evidence comes from a study of adolescents that showed no differences in depressive symptoms at baseline, but those that were identified as established smokers had twice the risk of developing depressive symptoms when assessed four years later (Choi et al., 1997). Research has also indicated that adolescent smokers have cognitive impairments compared to non-smoking controls. These deficits are observed both at baseline (i.e. nicotine on board with smokers) and are exacerbated during nicotine withdrawal (Jacobsen et al., 2005). This is an important consideration because changes in both affect and cognition during nicotine withdrawal are often cited as reasons to continue smoking. Thus, nicotine abuse during adolescence may lead to depressive symptoms and cognitive impairments later in life, further increasing the probability of continued use in adulthood.

Another important contributing factor to the detrimental effects of adolescent nicotine abuse may be stress. In adolescents, stress plays a vital role in the initiation of smoking in previously non-smoking individuals (Byrne et al., 1995). This is problematic because adolescents also report higher perceived levels of stress relative to children and adults, and stress during adolescence can lead to emotional dysregulation in adulthood (Compas et al., 1987; Pine et al., 2002; Green & McCormick, 2013). Thus, stress during adolescence can lead to the initiation of smoking, which not only increases

the likelihood of nicotine dependence throughout the lifetime, but could also augment cognitive and affective impairments resulting from adolescent nicotine use.

Although there is evidence to suggest adolescent smoking creates emotional dysregulation and cognitive impairments later in life, it is nearly impossible to form a cause and effect relationship in clinical research. For example, although adolescent smokers show cognitive deficits compared to non-smoking age matched controls, we cannot conclude that smoking is the cause of these impairments. Rather, it could be that those with observable or latent learning impairments are also more likely to smoke. Additionally, the link between early life stress and the development of adult depression is well established and occurs in the absence of nicotine use (Pine et al., 2002; Lupien et al., 2001; Heim & Binder, 2012). This is problematic because mood disorders such as depression have also been linked to cognitive impairments (Harvey et al., 2005; Austin et al., 2001). Thus, the combination of adolescent stress and adolescent nicotine could increase the risk of developing both cognitive and affective deficits later in life. The advantage of using a non-human animal model is that we can control for prior life experiences and examine cause and effect relationships. In fact, research from our lab indicates that adolescent nicotine exposure causes deficits in fear response learning that can be observed well after the drug treatment ends and that last into adulthood in mice (Portugal et al., 2012). Adult animals undergoing the same treatment conditions and tested at the same time delay after cessation of nicotine treatment do not display the same deficits.

In clinical studies, a link between adolescent stress and the initiation of smoking during adolescence has been established (Byrne & Mazanov, 2003). In

addition, the association between adolescent nicotine abuse and later mental health issues has also been documented (Brook et al., 2004). However, there could be other factors that mediate these relationships and using a non-human animal model helps to eliminate the possibility of extraneous factors influencing the relationship between adolescent smoking and adolescent stress and the development of later cognitive and affective impairments. Unfortunately, there is a paucity of research that examines the effects of adolescent nicotine and adolescent stress on adult behaviors in animal models. Given the role stress plays in mediating smoking behaviors in adolescence, and the development of cognitive impairments as a result of adolescent nicotine treatment, it is important to examine the interaction of adolescent nicotine and adolescent stress on deficits in fear response learning in adulthood (AIM 1), how to reverse these deficits (AIM 2), and examining if adolescent nicotine treatment leads to changes in measures of depression and anxiety (AIM 3).

CHAPTER 2

EXPLORING THE IMPACTS OF STRESS AND NICOTINE ON HIPPOCAMPUS-DEPENDENT BEHAVIOR

Rationale

Stress during adolescence has been identified as a risk factor for the initiation of tobacco use (Byrne, Byrne, & Reinhart, 1994). This is problematic because clinical studies have shown that daily stressful events occur more frequently and are perceived more negatively in adolescent humans compared to adults and children (Rahdar & Galvin, 2014). This, of course, is troubling because other researchers have established that a buildup of daily stressors, such as homework and interpersonal conflict with peers, has been linked to behavioral problems and risk for development of psychopathologies, including depression, which can exacerbate cognitive deficits (Dumont & Provost, 1988). Unfortunately, both adolescent stress and adolescent nicotine use impact regions critical for learning and memory. For example, chronic restraint stress (CRS), leads to distinct changes in behavior and cell morphology in male and female adolescent rats. At the end of the chronic restraint procedure, adolescent rats displayed decreased sucrose preference, an indicator of anhedonia, a symptom of depression (Eiland et al., 2012). In addition, adolescent rats showed decreases in apical dendrite length in the CA3 of the hippocampus and PFC, and increases in the BLA. Thus, stressed adolescents display depressive-like behaviors in addition to the patterns of alteration in the apical dendrites in areas associated with learning and memory. Moreover, research examining the impact of adolescent stress on learning and memory suggests chronic stress in adolescent male rats

resulted in memory impairments that are not seen in adults and mirror the changes in dendritic morphology. That is, stressed adolescent rats showed impairments in contextual fear conditioning, paralleling the dendritic atrophy observed in the hippocampus, and enhancements in auditory cued conditioning, paralleling the increases in apical dendrite length in the amygdala (Smith et al., 2006; Bergstrom et al., 2010). Furthermore, recent research has shown adolescent nicotine causes long-term alterations in the hippocampus (Trauth et al., 2000; Abreu-Villica et al., 2003) and persistent deficits in hippocampus-dependent learning (Spaeth et al., 2010; Portugal et al., 2012). However, there is a paucity of research examining the impact of both nicotine and stress exposure during adolescence on learning in adulthood.

In humans, stress experienced during the adolescent period is positively correlated with the risk of developing mood disorders such as depression later in life (Compas, Orosan, & Grant, 1993) and adolescent nicotine use is associated with both the development of adult depression and emerging cognitive deficits in adulthood (Heim & Binder, 2013; Jacobsen et al., 2005). However, studies that examine the combined impact of adolescent stress and adolescent nicotine are lacking. Thus far it has been determined that stress during adolescence augments the rewarding properties of nicotine and alters behavioral responses to nicotine later in life. Adolescent (p28) Sprague-Dawley male rats were exposed to a single trial of unpredictable foot shocks and twenty-four hours later were trained in a nicotine conditioned place paradigm (CPP) (Brielmaier et al., 2012). Foot shocks increased the time spent in the nicotine-paired side compared to non-stressed adolescent rats trained with nicotine. Further, this effect was blocked by systemic administration of CP-154,526, a corticotropin releasing factor receptor-1 (CRF-

R1) antagonist. This suggests that the enhancement of nicotine CPP following acute stress is caused by activation of the stress system. It is possible that the elevation of stress hormones following acute stress and subsequent nicotine treatment enhanced the associative learning rather than the nicotine reward, but this was not tested in the original study. This presents a unique problem-- if an adolescent is stressed and turns to smoking to alleviate his or her negative affect, they may find it far more rewarding than in a non-stressed situation, thereby increasing their risk of continued tobacco use. However, more pertinent to the current experiments is whether persistent deficits in hippocampus dependent tasks result from the combined effects of chronic adolescent nicotine and acute adolescent stress.

The overall purpose of AIM 1 was to determine if an interaction of stress and nicotine during adolescence causes long-term impairments in contextual fear. Important to this aim, we first characterized any changes in the stress response system, evaluated using corticosterone (CORT) concentration, a glucocorticoid, as the dependent measure, as a function of shipping stress and shipping stress combined with nicotine during adolescence. Thus, AIM 1 is broken down into three experiments. The first experiment determined if differences in corticosterone (CORT) concentration existed between shipped mice and bred mice in an age-dependent manner. Follow-up analysis sought to determine if shipping stress caused long-term changes in baseline CORT concentrations at different time points in an age-dependent manner. Finally, another cohort of adolescent mice was use used to see if nicotine administered during adolescence caused changes in baseline CORT levels at different time points. The second experiment sought to determine if an interaction of stress and nicotine during early adolescence and late

adolescence, but not adulthood, would result in impairments in contextual fear learning. The third experiment tested if inducing stress prior to administering chronic nicotine in adolescence or adulthood would result in impairments in contextual fear learning.

Methods

Experiment 1 Methods

Subjects and experimental groups

To compare CORT levels in shipped mice versus bred mice, whole blood was collected from shipped C57BL/6J male mice (Jackson Laboratory, Bar Harbor, ME; n=8-10 per group) at p31 or p47 one hour after arriving at our facility and from C57BL/6J mice bred in house (n = 8-10 per group) at p31 or p47. A separate cohort of mice was used to compare the long-term effects on stress response following shipping (n=8-10 per group). Blood was collected from adolescent and adult mice beginning at p31 or p47 respectively immediately following arrival in our facility, one week after arrival in the facility, and 6 weeks following arrival to our facility. These time points correspond to experimental time points for drug administration and behavioral testing. Finally, a separate cohort of only adolescent mice (n=8-12 per group) was used to determine if shipping stress and nicotine affected stress response following chronic nicotine or saline (n=8-12 per drug), withdrawal from chronic nicotine (n=8-12 per drug), and a thirty-day drug wash out period (n=8-12 per drug). These time points reflect experimental times for drug administration and behavioral testing.

Drug preparation and administration

For chronic experiments, nicotine (Sigma, St. Loius, MO; freebase, 12.6mg/kg/day) or saline drug was administered by mini osmotic pumps (Alzet), for a

period of 12 days. This dose was used because previous work has shown this raises cotinine levels to that seen in human smokers (Davis et al., 2006). Briefly, nicotine tartrate was dissolved in physiological saline and the amount was adjusted based on body weight of mice. Mini pumps were filled with this solution and implanted subcutaneously through a small incision on the back side of the mouse following procedures outlined on previous publications (Davis et al., 2006; Portugal et al., 2012). After twelve days, mini pumps were removed. All surgical procedures were conducted in a sterile environment with sterilized tools.

Blood collection and ELISA

To compare corticosterone (CORT) levels in shipped mice versus bred in-house mice, whole blood was collected from shipped mice at p31 or p47 and bred mice at p31 or p47. Blood was collected either 1 hour after arriving at our facility and being placed into a homecage (shipped mice) or 1 hour after being transferred to a new cage (bred mice) to control for novelty effects. Approximately 300-500uL of whole blood was collected by cardiac puncture following CO_2 euthanasia.

To further compare CORT levels in shipped adolescent and shipped adult mice whole blood was collected from the naive mice at three time points: immediately after arrival to our facility, one week after arrival at our facility, and 6 weeks after arrival at our facility. Blood was collected by using a 4mm lancet (Goldenrod) to pierce the submandibular area of the cheek and approximately 200-300uL was collected into an eppendorf tube.

A final cohort of mice was used to compare CORT levels in adolescence mice following chronic saline or nicotine treatment, 24 hours following withdrawal, or

following a 30-day drug wash out period, whole blood was collected through cardiac puncture following Avertin overdose.

For all experiments, blood was collected into eppendorf tubes and allowed to clot for 30 minutes. Afterwards, blood was spun down at 5,000rpm for 10 minutes to separate whole blood and serum. The serum was collected and transferred to new tubes and stored at -20C until ready for analysis. ELISA methods for evaluating corticosterone were conducted using the manufacturer's instructions (ImmunoDiagnostics). Briefly, samples were diluted 1:10 with sample buffer and all controls, standards, and samples were loaded onto a 96-well plate coated with antibody followed by 100uL of enzyme conjugate and incubated overnight at 4C. The plate was then washed three times, loaded with a TMB substrate and the reaction was stopped using hydrochloric acid. Mean absorbance rates were collected using a plate reader and software (Magellan). The kit used had an analytical sensitivity of 0.55ng/mL and the Intra-assay precision was $\leq 7\%$ and the inter-assay precision was $\leq 9\%$.

Statistical Analysis

To compare CORT concentrations in shipped versus bred mice separate independent samples t-tests were used for each adolescents and adults, as each age cohort was run on a separate ELISA plate. To compare differences in CORT concentrations in shipped adult and shipped adolescent mice at various time points following shipping stress a 2x3 repeated measures ANOVA was used with two levels of age (adolescent and adult) and three levels of time (immediate, 1 week, 6 weeks). To assess differences in adolescent mice treated chronically with nicotine or saline each time point (chronic,

withdrawal, 30 day) used an independent samples t-test was used comparing nicotine to saline treated mice as a separate ELISA plate was used for each time point.

Experiments 2 and 3 Methods

Fear conditioning

In both experiment 2 and experiment 3 mice were trained and tested in a contextual fear conditioning paradigm that allowed measurement of both contextual-based (hippocampal-dependent) (Logue et al., 1999; Kim & Fanselow, 1992) and cued-based memory (hippocampus-independent) (Philips & LeDeoux, 1992) and has been used extensively (Gould & Higgins, 2003; Davis et al., 2005; Portugal et al., 2012). Freezing, defined as lack of movement except for respiration, was measured for the dependent measure throughout the procedure. Freezing behaviors were assessed using a time-sampling method where every 10s mice were observed for 1s and scored as either freezing or active.

Apparatus

Mice were trained and tested in four identical chambers (17.78 cm × 19.05 cm × 38.10 cm) that were housed in sound attenuating boxes (Med-Associates). The front, back, and top chamber walls were Plexiglas, and side walls were stainless steel. The floors of the chambers were composed of metal rods that were connected to a shock generator and scrambler. Speakers were attached to the right wall of each chamber, and were used to administer the white noise CS (85 dB white noise). Ventilation fans provided air exchange and white noise (69 dB), and were mounted on the right wall of

each sound attenuating box. A computer connected to the chambers used Med-PC software to control stimulus administration. Testing for delay cued fear conditioning occurred in four identical altered chambers (20.32 × 22.86 × 17.78 cm) that were housed in sound attenuating boxes and located in a different room from the training chambers. The side walls of the chamber were made of aluminum, and all other walls were composed of Plexiglas. The chamber floors were covered in white plastic. Speakers for delivering the CS (85 dB white noise) were mounted on the left wall of each chamber. The background of the sound attenuating chambers differed in color from training chambers, and a vanilla extract olfactory cue was added to further distinguish these chambers from the training chambers. The apparatus was cleaned with 70% isopropyl ethanol before and after each mice.

Procedure

Mice were trained and tested in a contextual fear conditioning based off of the original protocol outlined by Gould and Higgins (2003). On the first day, mice were trained over a 5.5 minute session divided into CS-US pairings. For the first 120s, mice were allowed to freely explore the chambers and baseline freezing was assessed. Afterwards, a co-terminating conditioned stimulus (CS) (30s, 85dB white noise)—unconditioned stimulus (US) (2s, 0.57mA foot shock) was presented. After an additional 120s, a second CS-US pairing was presented. The session ended after an additional thirty seconds after which mice were removed and placed back in their home cage.

Twenty-four hours after training, mice were placed back in the training chamber with freezing to the context assessed over 5 minutes using a time-sampling method where freezing behavior was scored once every ten seconds. Freezing was defined as the lack of

movement other than respiration. After a short delay, cued fear conditioning was evaluated in different chambers with a solid floor and an additional olfactory cue (Vanilla Extract, Acme Markets). Sessions lasted 6 minutes, with the first 180s uninterrupted with and freezing was assessed with no cue presentation. For the second 180s, freezing was assessed during the presentation of the CS.

Drug Preparation

For experiment 2 mice were treated chronically with nicotine (Sigma, St. Louis, MO) or saline beginning at either p38 (adolescent) or p54 (adult) through the use of mini osmotic pumps (Alzet), designed to deliver 12.6mg/kg/day (freebase) for a period of 12 days. This dose was used because previous work has shown this raises cotinine levels to that seen in human smokers (Davis et al. 2006). For experiment 3, mice received nicotine through mini pump or underwent sham surgeries at the same time as pump implantation and pump removal. Briefly, nicotine tartrate was dissolved in physiological saline and the amount was adjusted based on body weight of mice. Mini pumps were filled with this solution and implanted subcutaneously through a small incision on the dorsal side of the mouse following procedures outlined by previous experiments (Davis et al., 2005; Gould et al., 2012). After twelve days, mini pumps were removed. All surgical procedures were conducted in a sterile environment. For both experiments, thirty days after pump removal mice were trained in contextual fear conditioning and tested 24 hours later.

Experiment 2: Comparing In-house Bred Mice and Shipped Mice

Subjects and experimental groups

Subjects were C57BL/6J male mice that were non-littermates either acquired from a vendor (Jackson Laboratory, Bar Harbor, ME) and shipped to our facilities in

Philadelphia, PA or bred in our mice facilities from breeders obtained from Jackson Laboratory. Shipped mice arrived to our facility as adolescents or adults, at either post-natal day 16 (p16) with dams, post-natal day 31 (p31) or post-natal day 47 (p47). Mice arriving at p16 were weaned from their dams at p21 into non-littermate groups of four. Mice obtained from breeding were bred in harem groups (2 females and 1 male) and separated out as soon as one female was visibly pregnant. Mice bred in our facility were weaned at p21 into same-sex pairs of 4. Mice obtained from shipping were also housed in pairs of 4. All mice received ad libitum access to food and water and all procedures occurred between 10:00AM and 5:00PM during the light phase of a 12h light/dark cycle. Behavioral and surgical procedures were approved by the Temple University Institutional Animal Care and Use Committee.

For the experiment examining the effects of shipping stress and nicotine on cognitive functioning, three age groups (early adolescent, late adolescent and adult) were divided into two drug groups (saline versus nicotine) and two stress conditions (shipped versus bred) for a total of 8 groups or 4 groups per age. Ages reflect the time nicotine or saline administration started. Thus, each age group had the following groups: 1) Bred in-house receiving saline (BRED-SAL; n = 8-16), 2) bred in-house receiving nicotine (BRED-NIC; n = 8-16), 3) shipped receiving saline (SHIP-SAL; n = 8-16), and 4) shipped receiving nicotine (SHIP-NIC; n = 8-16).

Statistical Analysis

Separate 2 [drug: saline versus nicotine] × 2 [stress: shipped versus bred] ANOVAs were used to analyze freezing scores for each age group corresponding to when mice began chronic nicotine administration. Significant main effects and

interactions were followed up with simple main effects, where appropriate. Results were considered significant at $p < .05$ and all data is presented as mean +/- the standard error of the mean.

Experiment 3: Induced stress

Induced Stress Paradigm

Subjects were male C57BL/6J mice obtained through breeding in our animal facility in Weiss Hall. In order to establish a stress protocol similar to shipping stress, adolescent mice were subjected to series of laboratory stressors and blood was collected to analyze CORT concentrations. Six weeks later mice were tested for deficits in contextual fear. The purpose of this was to create a stressor that produced comparable CORT elevations to age-matched shipped mice and did not create learning deficits on its own in adolescent mice. Adolescent mice (p31) were subjected to one of 6 stress conditions and CORT concentration was measured using blood serum, obtained through submandibular puncture detailed previously, 1 hour following stress. Six weeks following stress mice were trained and tested in the same fear conditioning model listed above. Stressors used were restraint stress or social instability stress or combination of restraint stress and social instability stress. Table 1 details results.

Stress Protocols

Restraint Stress: Mice were placed in 50mL conical tubes (with air holes) for a duration of 30 minutes, once a day, for one or two days. The duration and apparatus have been used in many previous studies. (Li et al., 2011; Laroche, 2009; Jorgensten, 2008; Isgor et al., 2004)

Social Instability Stress: Mice were isolated for 1 hour and then placed with a new cage mate for one or two days. After isolation, mice were placed with a same-sex novel cage mate in a new homecage until isolation and cage changing on the following day. Mice were placed in the same cage with no barriers and no two mice were paired together for more than one day (for review see McCormick, 2010; Saavedra-Rodriguez, 2013).

Combination Stress: Combination of restraint stress or social instability stress occurred with one stressor in the morning and one in the afternoon for 1 or 2 days, counterbalanced across groups.

Replicating the shipping stress learning deficit

Subjects and Experimental Groups

Subjects were male C57BL/6J mice obtained through breeding in our animal facility in Weiss Hall. Subjects were either adolescent (p31) or adults (p47) at the time of stress and received either nicotine or sham surgeries at p38 and p54, respectively. Induced stress using this paradigm occurred only in late adolescents and adults as the early adolescent group is not yet weaned by the time stress would occur. Each age group had 4 groups comprised of two stress conditions (induced stress or home cage controls) and two drug conditions (nicotine or sham surgeries). Thus, each age condition had the following groups: 1) sham surgeries and no stress (Sham-NoStress; n = 8-12), 2) sham surgeries and stress (Sham-stress; n = 8-12), 3) nicotine and no stress (Nic-NoStress; n = 8-12), and 4) nicotine and stress (Nic-Stress; n = 8-12).

Induced stress

The purpose of this experiment was to determine if a stress paradigm that did not cause persistent deficits in the contextual fear paradigm and elevated CORT levels to

similar levels seen in shipped adolescent mice would replicate the learning deficits following shipping stress and nicotine administration in adolescent mice. The induced stress model included two days of one stressor in the morning and one stressor in the afternoon for a total of 4 stress bouts over a period of 48 hours. Stressors used in this paradigm were a 30 minute restraint stress and 1 hour of social isolation followed by new cage partners. Each age group had 4 conditions comprised of 2 stress conditions (induced stress or home cage controls) and 2 drug conditions (nicotine or sham surgeries).

Statistical Analysis

To compare the effects of varying stressors to no-stress control mice on CORT elevation and freezing scores, separate one-way ANOVAS were used for each dependent measure. Separate 2 [drug: sham versus nicotine] x 2 [stress: induced versus none] ANOVAs were used to analyze freezing scores for each age group corresponding to when mice began chronic nicotine administration. Significant main effects and interactions were followed up with simple main effects, where appropriate. Results were considered significant at $p < .05$ and all data is presented as mean +/- the standard error of the mean. Finally, Pearson's correlation was used to see if there was a significant relationship between CORT levels following adolescent stress and freezing scores in adulthood.

Results

Experiment 1: Comparing CORT concentrations

CORT analysis: Shipped versus bred

When comparing shipped mice to mice bred in house 1 hour after arrival or 1 hour after being placed in a new home cage respectively, only adolescent mice shipped to our facility show elevations in corticosterone ($t(20)=5.35$, $p < .001$) compared to age-matched controls bred in our facility. There were no significant differences between adult mice shipped to and age-matched controls bred in our facility. Thus, adolescent mice were more sensitive to the acute effects of shipping stress than age-matched controls (see figure 1).

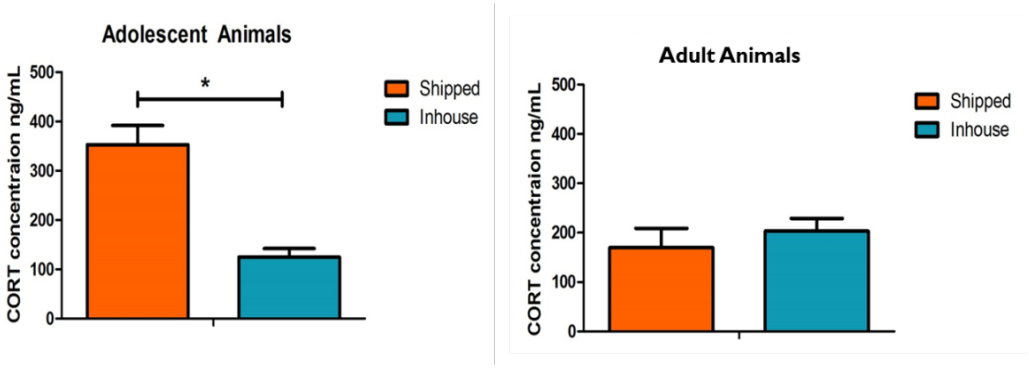


Figure 1: Comparing CORT concentrations in shipped and bred mice. Each bar represents mean CORT concentrations and the error bars show standard error of the mean. * $p < 0.05$

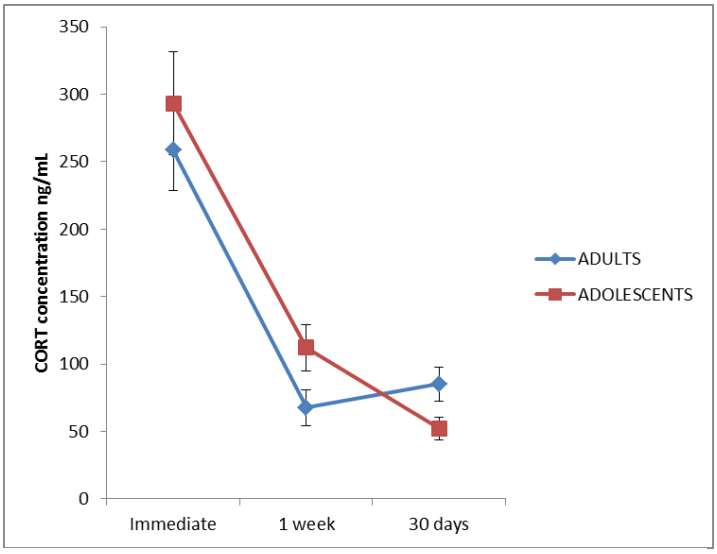


Figure 2: CORT concentrations following shipping stress. Adolescent (n=6-8) and adult (n=6-8) mice. Each point represents the mean CORT concentration and error bars are SEM.

CORT analysis: Adolescent versus adult

When comparing shipped adolescent to shipped adult mice at three time points there was a significant interaction of time x age ($F(1,17) = 7.44$, $p < 0.01$). However, post-hoc tests at each time point show no significant difference between ages. Shipping stress does not produce persistent age-dependent changes in the stress response system (see figure 2).

CORT analysis: Chronic nicotine

There were no significant differences in CORT levels between adolescent mice receiving nicotine or saline at 24 hours of withdrawal of after the 30 day washout. There was a trend ($p = 0.07$) comparing chronic nicotine and chronic saline with chronic nicotine administration leading to lowered CORT concentrations (See figure 3).

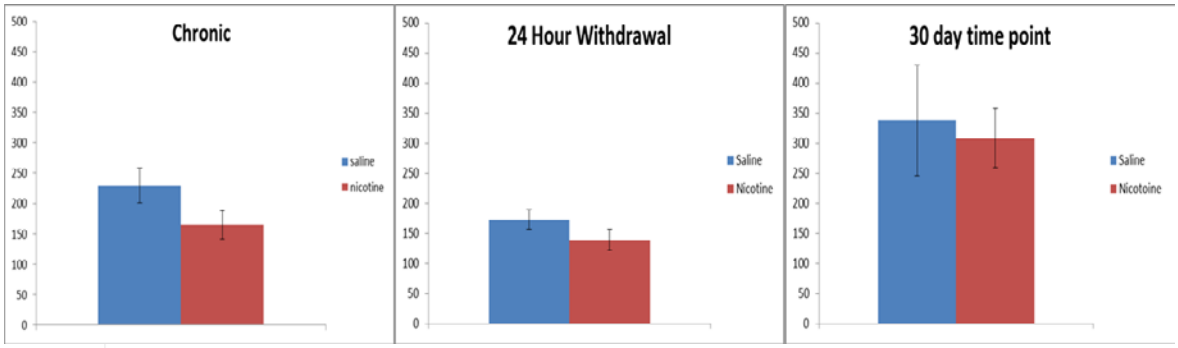


Figure 3: Comparing CORT concentration in adolescent mice after saline or nicotine. Bars represent mean CORT concentration at time point during or following 12 days of chronic nicotine administration, error bars represent SEM.

Experiment 2: Comparing bred in-house mice and shipped mice in contextual fear

Separate 2x2 ANOVAs (Drug [saline versus nicotine] x Stress [shipped versus bred]) were used for each age group for freezing counts for the following time points during fear conditioning: baseline, context, preCS and CS. Results for fear conditioning are broken down by age at which nicotine was administered.

Experiment 2: Examining the combined effects of stress and nicotine on contextual fear learning

Early adolescence. In the early adolescent cohort, mice that were shipped to our facility and received chronic nicotine demonstrated impaired contextual fear learning (see figure 4). There were no group differences in baseline freezing. There was a significant

interaction ($F(1,56)=6.99$, $p < .01$) when analyzing freezing to the context. To follow-up the significant interaction, simple main effects were run. In mice that received saline there were no differences between shipped mice (M=11.81) or bred in-house mice (M=13.42). However, in mice that received nicotine there was a significant difference between shipped mice (M=4.38) versus bred-inhouse (M=12.36), ($F(1,56) = 23.59$, $p < .001$)). Thus, SHIP-NIC mice had significantly lower freezing scores to the context than BRED-NIC, indicating the interaction of stress and nicotine during early adolescence impairs hippocampus-dependent learning. In the preCS time point, there were no main effects of drug or stress but there was a significant drug x stress interaction ($F(1,56) = 4.06$, $p < .05$). Simple main effect analysis revealed that in shipped mice there were no differences in mice receiving nicotine (M=.81) and saline (M=.94). However, there was significant difference in mice that were bred in house ($F(1,56) = 5.80$, $p <.05$) whereby nicotine treated mice had lower freezing scores (M=.43) than saline treated mice (M=1.29). For the CS time point there was a main effect of stress condition ($F(1,56) = 10.23$, $p < .01$) whereby mice that were shipped had significantly lower freezing to the cue (M = 10.89) than mice bred in-house (M=13.19). Taken together, this indicates that stress and nicotine during early adolescence interact to alter hippocampus functioning leading to deficits in contextual fear conditioning and that stress during the early adolescent period interferes with hippocampal-independent learning and drug treatment may play a role in this difference.

Late adolescence. In the late adolescence group, mice that received nicotine and experienced shipping stress had deficits in contextual fear learning (see figure 4). There were no differences between groups for baseline freezing. There was a significant

interaction between drug and stress ($F(1,61) = 4.66$, $p < .05$). Simple main effects were used to follow up the significant interaction. In the bred mice, there was no significant effect of drug and mice that received saline (M=12.33) had similar freezing scores to the context than mice that received nicotine (M=11.6). In contrast, shipped mice that received nicotine (M=8.06) had significantly lower freezing scores to the context compared to mice that received saline (M=13.25), ($F(1,61) = 12.48$, $p < .001$). There were no significant difference between groups at the preCS or CS time point. Thus, in late adolescent mice, the SHIP-NIC condition resulted in contextual fear learning indicating impaired hippocampal functioning but the SHIP-NIC condition demonstrated the same level of freezing as the other three groups for the hippocampus-independent cued fear conditioning.

Adulthood. In the adult group there were no effects of drug or stress nor an interaction between drug and stress freezing during baseline, contextual, preCS or CS time points. (see figure 4). Taken together this indicates that mice that were stressed and treated with nicotine during adulthood do not show learning deficits in the hippocampus-dependent and hippocampus-independent learning paradigms.

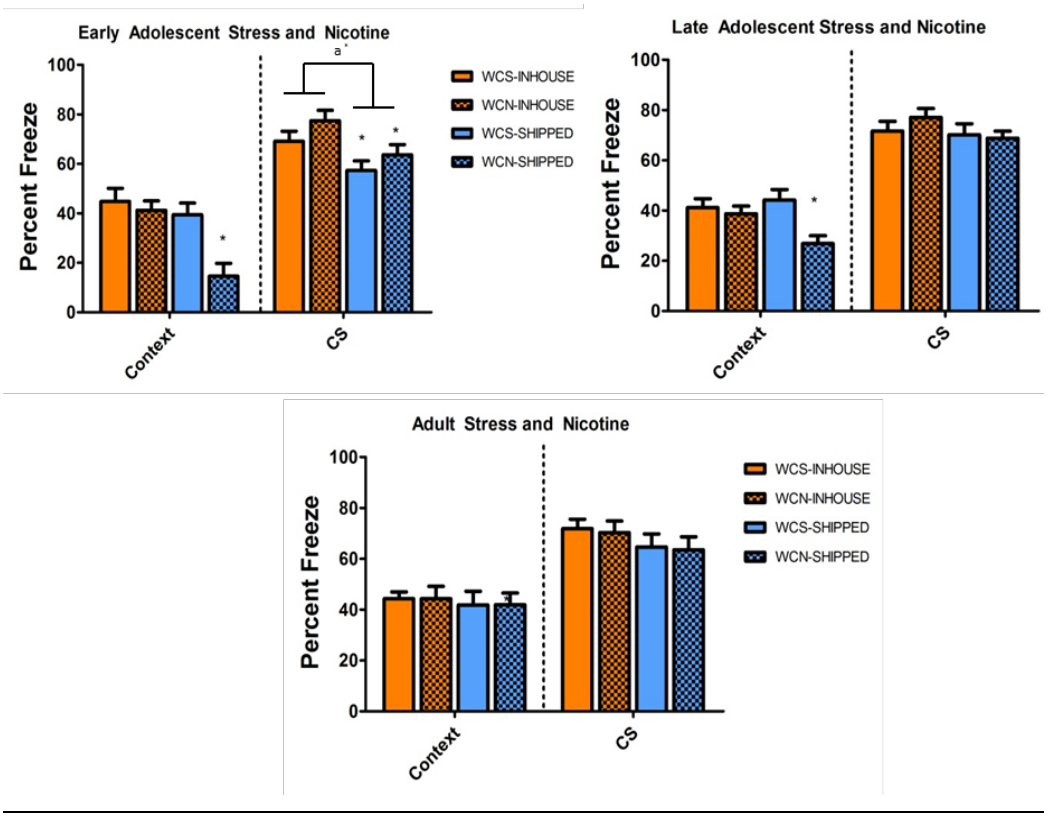


Figure 4: Interaction of stress and nicotine on contextual and cued learning. Early adolescent, late adolescence, and adult mice tested in contextual and cued conditioning following a 1 month protracted abstinence. Bars represent mean percent freezing and error bars are SEM. WCS= withdrawal from chronic saline WCN = withdrawal from chronic nicotine * $p < .05$ significant simple main effect compared to stress condition; a*= $p < .05$, significant main effect

Experiment 3: Replicating the effects of shipping stress with an induced stress paradigm

In the induced stress experiments, a one-way ANOVA was used to compare CORT concentrations, contextual freezing, and cued freezing. Like experiment 2, adolescent (p38) and adult (p54) cohorts freezing was analyzed using separate 2 × 2 ANOVAs (drug [controls versus nicotine] x stress [stress versus no stress] for contextual fear and cued fear. Results are separated by the age at which mice received nicotine.

Induced Stress Procedure

When comparing CORT concentrations using a one-way ANOVA there was a significant difference between groups ($F(4,29) = 6.139$, $p < 0.05$). Bonferroni post-hoc tests indicate CORT elevations in the 2 day combination stress compared to no stress ($p < 0.05$). Additionally, when comparing contextual fear conditioning and cued conditioning between stressors and controls there were no differences. Thus, the 2-day combination stressor was used to mimic the shipping stress condition, to account for both the physical stress (restraint) and social stress (social instability) that mice encounter during transportation stress (Laroche, 2009) (see table 1). There was no significant correlation between CORT concentrations and later contextual freezing scores ($p > .05$) indicating that early environmental stress does not cause learning deficits.

Comparing Corticosterone (ng/mL) concentrations				
Comparison from Experiment 1	Age at arrival	CORT (ng/mL)		
		Shipped	Bred	
	p31	353.16 ± 38.81 **	124.76 ± 17.72	
	p47	169.8 ± 39.32	203.37 ± 25.90	
**Indicates significant difference from bred controls				
Comparing Corticosterone (ng/mL) concentrations and Freezing scores				
	Age time of stress	CORT (ng/mL)		Percent Freezing to Context
Comparison from Experiment 3	p31	Control	184.4 ± 40.29	42.20%
		2 day restraint	431.81 ± 75.48**	32.10%
		2 day social	357.99 ± 73.62	35.83%
		1 day combined	168.77 ± 54.63	42.37%
		2 day combined	436.97 ± 33.62**	43.67%
**indicates significant difference from no-stress controls, p < .05				

Table 1: Comparing corticosterone and contextual fear learning across conditions. Mean CORT levels are displayed from mice in experiment 1 that were either shipped or bred in our facility. Mean CORT levels and freezing to a context paired with a shock are displayed. Based on these results, the 2 day combined stress utilizing restraint and social instability was used for all induced stress experiments. ** indicated significance at $p < .05$.

Late adolescence: In the late adolescent cohort, mice that underwent the induced stress paradigm and administered chronic nicotine had deficits in contextual fear learning (see figure 5). There were no group differences in freezing behaviore at the baseline time point. There was a significant interaction ($F(1,32) = 4.40$, $p < 0.05$). Follow-up simple main effects indicate that there were no differences in the no-stress condition between saline and nicotine mice. However, in the stressed condition, saline mice froze to the context significantly more (M=11.83) than nicotine mice (M=5.63), $F(1,32) = 20.12$, $p < 0.001$. There were no group differences in the preCS freezing or cued freezing indicating

the learning impairments resulting from the interaction between stress and nicotine during adolescence is specific to the hippocampus-dependent learning.

Adulthood. In the adult cohort there were no group differences in freezing during baseline, contextual, preCS or time points (See figure 5). Thus, there were no interactive effects of stress and nicotine on hippocampus-dependent or hippocampus-independent

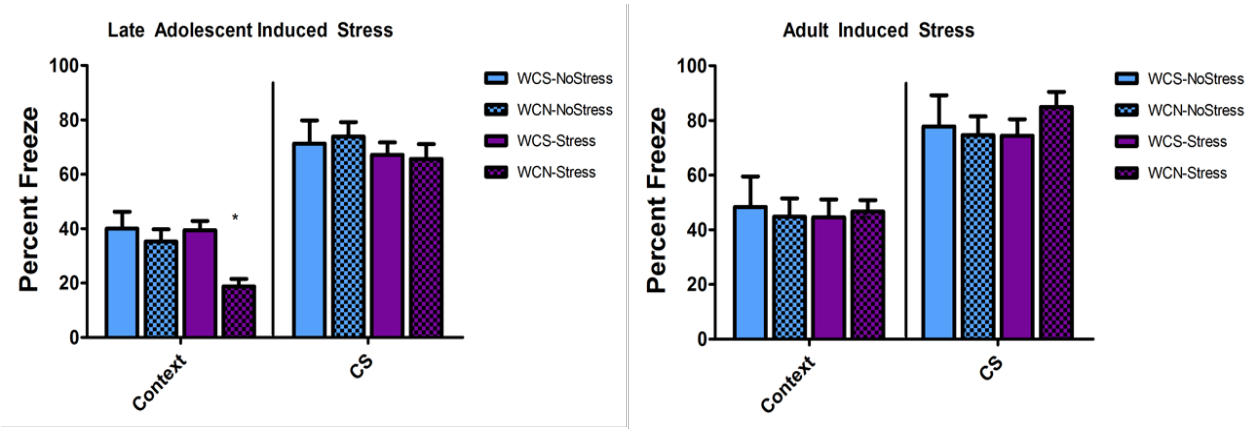


Figure 5: Induced stress and nicotine on fear learning. Late adolescent and adult mice tested in contextual and cued conditioning following the induced stress paradigm after 1 month protracted abstinence. Bars represent mean percent freezing and error bars are SEM. WCS=withdrawal from chronic saline WCN=withdrawal from chronic nicotine. * $p < .05$ following significant simple main effect within stress condition

learning.

Discussion

The major findings from these experiments are that the interaction of stress and chronic nicotine during adolescence leads to impaired hippocampal-dependent learning. This study also found that adolescent mice experience shipping stress differently than adult counterparts as elevations in CORT were higher in shipped adolescents than bred adolescents, shipped adults, and bred adults. Shipping stress did not affect baseline levels of CORT at later time points indicating that shipping stress alone does not cause persistent changes in the stress response system. Further, adolescent nicotine following shipping stress did not alter the stress response during chronic nicotine administration, withdrawal from chronic nicotine, or after a thirty day drug washout compared to saline controls. This indicates the combination of shipping stress and nicotine do not alter HPA function. Further, the studies utilizing the induced stress paradigm in an effort to replicate the shipping stress effects demonstrated the interactive effects of nicotine and stress were more sensitive in the adolescent epoch. Adult mice exposed to the induced stress and administered chronic nicotine did not display the same learning deficits. Thus, the results from these studies reveal stress, through shipping stress or a combination of laboratory stressors, combined with chronic nicotine during adulthood has an interactive deleterious effect on hippocampus functioning that manifests in adulthood.

This study builds on previous work that demonstrates chronic adolescent nicotine leads to deficits in contextual fear learning (Spaeth et al., 2010; Portugal et al., 2012). For example, adolescent rats given chronic nicotine treatment (3.0mg/kg) from P28-p42 showed deficits in contextual conditioning, evident by decreased licking suppression in a context previously associated with a shock (Spaeth et al., 2010). It is difficult to

determine if this effect is age-dependent as adult rats were not used for comparison. However, when examining contextual fear conditioning in adolescent and adult mice, it is clear that chronic nicotine exposure during adolescence causes long-term impairments in adult cognition (Portugal et al., 2012). Early adolescent (p23), late adolescent (p38), and adult (p54) mice were administered nicotine for 12 days and thirty days after nicotine cessation, mice were assessed in fear conditioning. Both early adolescent and late adolescent mice showed deficits in contextual fear conditioning while adult mice did not and cued conditioning remained unaffected in both age groups. Further, early adolescent mice showed deficits at both doses (8.8 and 12 mg/kg) of nicotine while late adolescents showed deficits only at the higher dose. These effects may be related to changes in CREB availability, as CREB expression was reduced in the hippocampus but increased in the cortex of early adolescent mice following chronic nicotine treatment and this effect was not seen in adult mice (Portugal, 2012). Thus, chronic nicotine during adolescence likely leads to changes in gene expression that contribute to learning deficits observed in adulthood. However, results from the present study indicate that learning deficits emerge as a function of stress during adolescence followed by chronic nicotine administration as nicotine alone during adolescence did not lead to learning deficits.

Data collected from this study suggests that an unaccounted source of stress in the form of shipping from one mice facility to another during critical periods of development combined with nicotine administration during adolescence may contribute to long-lasting alterations in contextual fear learning. These results indicate that adolescents show an augmented response to shipping stress compared to adults and that mice needed to undergo both stress and nicotine treatment during adolescence to display deficits in

contextual fear learning. Furthermore, most studies that indicate long-term deficits in attention (Counette et al 2010), learning (Portugal et al, 2012), or reward (Brielmaier et al, 2007) resulting from adolescent nicotine exposure had mice shipped to the laboratory location. Thus, it is likely that a combination of adolescent stress and adolescent nicotine results in aberrant behavior but this has gone unnoticed until recently and warrants further investigation of this apparent interaction.

Alterations in a wide range of behavior and elevations in CORT have been documented following transportation in adult mice with behaviors returning to normal after a week and CORT returning to baseline after a day (Tuli, Smith, and Morton, 1995). Results from this study suggest that while CORT elevations in adult mice occur immediately upon arrival to our facility, CORT returns to baseline after 1 hour of being placed in a new home cage. Adolescent shipped mice show an elevation of CORT immediately upon arrival, persisting to 1 hour after arriving to our facility compared to bred controls but returned to baseline 1 week following arrival at our facility, in support of Tuli et al. (1995). Furthermore, few studies have directly examined the behavioral and physiological effects of shipping during the adolescent period (Wiley & Evans, 2009; Ismail, Garas, and Blaustein 2011; Laroche et al., 2009). These studies unequivocally suggest that adolescence is especially susceptible to long-term alterations resulting from this type of stressor. For example, shipped Long-Evans adolescent male rats were more sensitive to the antinociceptive, hypothermic, and cataleptic effects of THC and were more sensitive to locomotor activity after receiving haloperidol, an antipsychotic used to treat schizophrenia, compared to bred Long-Evan controls (Wiley & Evans, 2009). Additionally, shipping during the adolescent period creates persistent impairments in

sexual receptivity in mice and the impairments were dependent on when the mice were shipped (Laroche et al., 2009). For example, female and male C57BL/6 mice had lowered levels of sexual behavior when shipped at 6 weeks but these effects were not present when mice were shipped as adults at 12 weeks. In a follow-up study, Laroche and researchers attempted to create the effects of shipping stress by exposing 6 week old mice bred in-house to either repeated restraint stress, food deprivation, a chronic variable stressor paradigm or injections of bacterial endocrine lipopolysaccharide (LPS). It was found that only the immune stressor (i.e. LPS injections) led to similar reductions in sexual behaviors in adulthood (Laroche et al., 2009b). Additionally, LPS injections at 3 weeks and beyond 6 weeks had no effect on adult reproductive behaviors. This indicates that the adolescent period between 4-6 weeks is especially vulnerable to long-term changes as a result of stressors.

Results from the current study are in agreement with adolescence being a vulnerable time for environmental factors producing alterations that impact adult behavior that may be exacerbated by adolescent nicotine. Adolescents tend to show more exaggerated responses to acute stressors and this supports the data that shipped adolescent mice had higher CORT elevations than shipped adult mice (Bahdar & Galvin, 2014; Allen & Matthews, 1997). Additionally, some research suggests that stress during the adolescent period impairs hippocampal-dependent learning in adulthood. Specifically, exposure to variable stressors beginning in early adolescence (p28) and continuing into early adulthood (p56) resulted in impairments in MWM when tested 3 weeks after the termination of stress (Isgor et al., 2004). This stress paradigm also led to a persistent down regulation of GR and MR receptors, which was observed from 1 week to 12

months after the termination of stress (Isgor et al., 2004). These changes were accompanied by volumetric reductions in the hippocampus, evident at the same time point (2004). Shorter stress duration during adolescence led to the same impairments in MWM performance compared to non-stressed controls when tested as adults (Avital and Richter-Levin, 2005). Other research suggests that isolation stress during adolescence impacts the acquisition of a spatial task, but after several days of training, stressed rats perform at comparable levels to non-stressed rats (Sandstrom & Hart, 2005). However, it is important to note that shipping stress and induced stress alone did not create deficits in contextual fear learning. This could be due to the fact that the stressors used in the present study were shorter in duration and thus did not produce learning deficits on their own. Rather, it was the combination of stress and nicotine during early adolescence and late adolescence that led to deficits in hippocampus-dependent learning. Further, there was not a significant correlation between adolescent CORT levels following the induced stress optimization and later adult fear conditioning, suggesting the deficits observed in contextual fear learning were not solely due to elevations in CORT. It is also important to point out that shipping stress did not alter baseline circulating CORT levels at later time points, indicating alterations in the stress system at these time points are not mediating the impairments in contextual fear learning. The lack of changes in baseline CORT, coupled with the fact that shipping stress and induced stress did not produce deficits on their own, indicates that the impairments in contextual fear learning are due to the interactive effects of nicotine and stress during adolescence.

Given the important finding that stress and nicotine act synergistically during adolescence to produce learning impairments in adulthood, it is important to review what

is known regarding the interactive effects of nicotine and stress. First, stress can lead to compulsive drug use and reinstatement of drug seeking behaviors, indicating activation of the stress before the introduction of drugs may increase the likelihood of developing dependence (Koob & Kreek, 2007; Liu & Weiss, 2002; Erb et al., 1996). In addition, acute nicotine injections elevate corticosterone (CORT), much like elevations observed after acute stress (Balfour, Khullar, & Longden, 1975). However, repeated administration of nicotine also leads to a suppression of the stress response, as chronic nicotine treatment leads to habitation of CORT levels and also attenuates CORT concentrations following an acute stressor (Faraday et al., 2005). Results from this study show a trend suggesting chronic nicotine administration during adolescence leads to reductions in CORT levels relative to saline controls. This coupled with the fact GR and MR receptors, which CORT binds to, were downregulated following adolescent stress may partially explain the observed deficit (Sterlemann et al., 2008). That is, since activation of GR receptors following contextual training are necessary to develop the contextual memory (Cordero & Sandi, 1998), and since GR receptors are downregulated following adolescent stress and stress reactivity is blunted following chronic nicotine administration, it is possible that adolescent stress and adolescent nicotine disrupt the stress response in such a way that acquisition of the context-shock association was disrupted in adulthood. Future work should test to see if circulating CORT levels following the shock presentation are reduced in mice exposed to stress and nicotine during adolescence.

The finding that stress and nicotine interact in such a way to create learning deficits is somewhat surprising considering that nicotine alone and stress alone did not

cause the same deficits in the paradigm used here. One explanation is that the nicotine dose used in these experiments is not large enough to create long-term deficits on its own; similarly, the stress used in these experiments may result in subclinical learning deficits that only emerge when combined with nicotine. Thus, it is possible that what we are modeling is a "two-hit" stress process, whereby the shipping stress followed by the administration of nicotine act synergistically to create alterations in the functioning of the hippocampus. Nicotine can activate the stress response (Balfour et al., 1975) which may play a role in the long-term hippocampal impairments resulting from the interaction of stress and nicotine. Thus, it is possible that stress during adolescence creates short-term alterations in hippocamapal cell structure, such as pyramidal cell atrophy and loss of neurogenesis (Isgor et al., 2004; Vyas et al., 2004) or alters GR and MR mRNA expression in the hippocampus (Sterlemann et al., 2008) but is reversed or compensated for over time. The addition of nicotine may make these changes more prominent or even irreversible. Future work will determine the specific action of stress and nicotine as it is still unclear how the stress and acetylcholinergic system interact to create long-term deficits.

CHAPTER 3

REVERSING ADULT LEARNING DEFICITS RESULTING FROM ADOLESCENT NICOTINE WITH DIETARY CHOLINE SUPPLEMENTATION

Rationale

The acetylcholinergic system plays a large role in learning and memory (Gold, 2003). ACh is synthesized through the combination of choline and acetyl CoA through the enzyme choline acetyltransferase (ChAt). Acetylcholine is stored in presynaptic vesicles until an action potential causes the release into the synaptic cleft. From there, acetylcholine can bind to cholinergic receptors including nicotinic acetylcholine receptors (nAChRs) or muscarinic acetylcholine receptors (mAChRs) (Graef et al., 2011; Abreu-Villica et al., 2011; Prado et al, 2002). Of importance, the hippocampus has a high concentration of nAChRs (reviewed by Gould & Leach, 2014) and since nicotine acts through nAChRs it is no surprise nicotine can modulate learning and memory. Specifically, acute nicotine can enhance (Gould & Lommock, 2003; Gould and Higgins 2003), while withdrawal from chronic nicotine can impair hippocampus-dependent learning in adult mice (Davis et al., 2005; Portugal et al., 2009). Further, chronic nicotine administration during adolescence leads to long-term deficits in hippocampus-dependent learning (Portugal et al., 2012). Thus, in order to alleviate long-term learning deficits it is important to understand how adolescent nicotine modulates the function of the cholinergic system.

The cholinergic system reaches maturation during adolescence, with receptor densities and projections reaching stable levels around mid-adolescence (p30) in rodents (Abreu-Villica et al., 2011). This may explain why adolescence is a vulnerable time

period for long-term learning impairments as a result of chronic nicotine use. Specifically, cholinergic development in rodents occurs mostly within the first three weeks of post-natal life, with stable levels of enzyme activity and projections reached by mid-adolescence. For example, ChAt activity reaches its peak by p30 and gradually decreases until stabilizing at p60 (Thal et al., 1992). Previous work has indicated that nicotine administered during adolescence leads to long-lasting reductions in cholinergic activity in the hippocampus, which may partially explain the persistent deficits in hippocampus-dependent learning following chronic adolescent nicotine administration (Trauth et al., 2000; Slotkin et al., 2008). In support of this, pilot data suggest that chronic nicotine administration during adolescence leads to decreased sensitivity to subsequent nicotine in adulthood (Holliday & Gould, in prep). That is, a higher dose of nicotine was needed to enhance contextual fear learning in adults that received chronic nicotine treatment during adolescence. Taken together, this suggests nicotine during the adolescent period leads to reduced sensitivity in the cholinergic system and the hippocampus is especially vulnerable to these alterations.

Previous research has indicated that administering choline, the necessary precursor to the synthesis of acetylcholine, during prenatal and postnatal periods facilitates spatial learning tasks in adulthood (Meck et al. 2008). Additionally, other research suggests that choline administration may be a useful intervention for ameliorating cognitive deficits that arise from drug exposure during critical periods of development (Velazquez et al., 2014). For example, choline supplementation following fetal alcohol exposure reverses cognitive deficits associated with alcohol exposure in utero (Hunt, 2012). In addition, in rats exposed to alcohol during gestation, choline

supplementation reversed the expected deficit in trace conditioning but not in delay conditioning, suggesting that the reversal effects of choline supplementation are limited to hippocampus-dependent learning (Thomas & Tran, 2012). One way choline may reverse these cognitive deficits is through increasing the synthesis of acetylcholine, a neurotransmitter heavily implicated in learning and memory tasks (Murai et al., 1994). Another way in which choline may act is by altering gene expression in regions of the brain that support learning and memory, such as the prefrontal cortex and hippocampus (Niculesu et al, 2006; Davison et al., 2009; Otero et al., 2012). For example, epigenetic changes have been observed that parallel improvements in learning tasks following alcohol exposure and choline administration during adolescence (Otero et al., 2012). This suggests choline helps to ease deficits resulting from chronic drug exposure. Finally, choline is an α7 agonist (Alkondon et al., 1997; Mike et al., 2000) and α7 receptors play a role in synapotgensis and axogenesis during adolescence which may help attenuate alterations in the cholinergic system as a result of chronic nicotine exposure during adolescence (Slotkin et al., 2004; Chan & Quik, 1993). Thus, this study wanted to test if chronic dietary choline supplementation following adolescent nicotine treatment would reverse deficits in hippocampus-dependent learning.

Methods

Subjects and experimental conditions

Male C57L/6J mice obtained from Jackson Laboratories (Bar Harbor, ME) at either post-natal day (p) p16 (early adolescent), p31 (late adolescent), or p47 (adulthood). Early adolescent mice were shipped with dams and weaned at p21. Each age had four experimental groups consisting of two drug conditions (nicotine or saline) and two diet

conditions (standard and choline supplemented). Thus, each age group had the following condiitons: 1) Saline-Standard (SAL-CHOW), 2) Saline-Choline (SAL-CHOL), 3) Nicotine-Standard (NIC-CHOW), and 4) Nicotine-Choline (NIC-CHOL).

Drug and Diet Methods

One week following arrival at our animal facility, mice were subcutaneously implanted with mini osmotic pumps to deliver saline or nicotine (Sigma, St. Louis, MO; freebase,12.6mg/kg/day) at p23, p38, and p54. Nicotine or saline was delivered continuously for 12 days at which time the mini pumps were removed. All mice underwent a 30 day drug-free period during which time mice were given ad libitum access to either standard mouse chow (LabDiet Mouse Chow 5015) or a choline supplemented diet (TestDiet; Richmond, IN). Standard mouse chow had 2000ppm (2g/kg) of choline and the TestDiet choline supplemented diet had 9000ppm (9g/kg), which was 4.5x greater than the standard diet. Previous work has demonstrated that this concentration of choline reversed many cognitive deficits associated with rat models of fetal alcohol syndrome and schizophrenia (Velazquez et al., 2013; Glenn, Adams & McClurg, 2012; Corriveau & Glenn, 2012). Following 30 days of a drug washout period and continuous access to either standard diet or choline supplemented diet, all mice were trained and tested in the previous mentioned contextual fear conditioning paradigm with access to their respective diets in between sessions.

Contextual Fear Conditioning

Apparatus. Mice were trained and tested in four identical chambers (17.78 cm × 19.05 cm × 38.10 cm) that were housed in sound attenuating boxes (Med-Associates, St. Albans, VT). The front, back, and top chamber walls were Plexiglas, and side walls were

stainless steel. The floors of the chambers were composed of metal rods that were connected to a shock generator and scrambler. Speakers attached to the right wall of each chamber administered the white noise CS (85 dB white noise). Ventilation fans provided air exchange and white noise (69 dB), and were mounted on the right wall of each sound attenuating box. A computer connected to the chambers used Med-PC software to control stimulus administration. Testing for cued fear conditioning occurred in four identical altered chambers (20.32 × 22.86 × 17.78 cm) that were housed in sound attenuating boxes and located in a different room from the training chambers. The side walls of the chamber were made of aluminum, and all other walls were composed of Plexiglas. The chamber floors were covered in white plastic. Speakers for delivering the CS (85 dB white noise) were mounted on the left wall of each chamber. The background of the sound attenuating chambers differed in color from training chambers, and a vanilla extract olfactory cue was added to further distinguish these chambers from the training chambers. The apparatus was cleaned with 70% isopropyl ethanol before and after each animal.

Procedure. On the first day of training, mice were allowed to freely explore the conditioning chamber for 120 seconds and baseline freezing was measured. Mice were then exposed to an auditory stimulus (CS, 85 dB white noise) lasting for 30 seconds that coterminated with a 2 second shock (US, 0.57mA, minimum level necessary for learning: Gould, 2003; Gould, Feiro, and Moore, 2004; Gould and Higgins, 2003; and Gould & Lommock, 2003). After this mice remained in the chambers for an additional 120 seconds and another CS-US pairing was presented. Mice remained in the cage for 30 seconds with the lights and fans on before being placed in their home cage. On the

second day, mice were placed in the same chamber and freezing behavior was scored for the next 5 minutes to measure contextual conditioning (context). One hour later, mice were placed in a different chamber and allowed to explore for 180s with freezing behavior assessed (preCs) before being presented with the auditory stimulus (CS) for another 180s.

Statistics

Separate 2 × 2 ANOVAs were ran with two drug conditions (saline versus nicotine) and two diet conditions (standard versus choline supplemented) for each measure. Main effects are presented with no additional post hocs since only two levels exist for each factor. Significant interactions were followed up with simple main effects since each factor has two levels. Results are separated by age at which mice received nicotine treatment.

Results

Early Adolescence

In the early adolescence cohort, mice that received nicotine and were placed on the standard diet displayed the expected deficits in during adulthood in contextual fear conditioning. Dietary choline reversed these deficits in nicotine treated mice. There was a significant interaction between diet and drug ($F(1,43) = 4.74$, $p < .05$) in contextual fear conditioning. Simple main effects revealed that there was significant difference in mice on the standard diet ($F(1, 43) = 7.21$, $p < .01$) such that mice treated with nicotine (M=7.27) had significantly lower freezing scores than mice treated with saline (M=11.92). There was no significant difference between saline-treated and nicotine-treated mice in the choline condition. This indicates that mice receiving nicotine during

early adolescence have persistent impairments in hippocampus function that are not apparent after supplemented dietary choline. For the CS, there were no main effects of drug and no interaction but there was a significant main effect of diet ($F(1,43) = 5.83$, $p < .05$). Mice that received the choline supplemented diet (M=12.13) had higher freezing than mice receiving the standard chow (M=10.43). There were no group differences in baseline freezing or freezing during the preCS, indicating the enhancement in cued learning as a result of choline diet administration were not due to changes in locomotor activity. This suggests that overall choline had different effects on different types of learning when administered in adolescence into adulthood and can ameliorate deficits in hippocampus-dependent learning (see figure 6).

Late Adolescence

In the late adolescent cohort mice that received nicotine and placed on the standard diet showed deficits in contextual fear whereas this effect was reversed in mice that received nicotine followed by dietary choline supplementation. There was a significant interaction, ($F(1,59) = 5.89$, $p < .01$) in contextual fear learning. Follow-up simple main effects indicate there was no significant difference in diet condition in mice receiving saline but there was a significant difference in diet condition in mice receiving nicotine ($F(1,59)=15.13$, $p < .05$). Specifically, mice in the NIC-CHOW (M=6.53) condition had significantly lower freezing scores than mice in the NIC-CHOL (M=12.06). This indicates that chronic nicotine treatment leads to deficits in contextual fear learning that are not observed following supplemental choline. Additionally, there were no differences at the preCS, indicating freezing to a novel context was similar in all

groups. Finally, there were no group differences in cued fear conditioning indicating hippocampus-independent learning was not altered.

Adulthood

In mice treated with nicotine and choline during adulthood (p54), there were no significant differences between groups in contextual fear conditioning indicating hippocampal-dependent learning was not altered by either drug or diet treatments. There were no differences in baseline freezing or freezing during the preCS. In cued conditioning there was a significant interaction between drug and diet condition ($F(1,48) = 7.16$, $p < .01$). Simple main effects indicate that within the standard chow condition, nicotine-treated mice (M=13.5) froze more to the cue than saline-treated mice (M=11.5) while there was no difference between drug treatment in the supplemented choline diet. This indicates that adult mice in the NIC-CHOW condition had enhanced cued fear conditioning, though this was not seen in adult mice that received adolescent nicotine in the NIC-CHOW groups.

Discussion

The purpose of these experiments was to determine if choline supplementation following chronic nicotine administration during adolescence would reverse adult deficits contextual fear learning. Specifically, early adolescent and late adolescent mice administered nicotine for 12 days and then given dietary choline supplementation (9g/kg) during the 30 day drug wash out period showed similar levels of adult contextual learning compared to saline and standard diet controls. In contrast, early and late adolescent mice that received nicotine and standard mouse chow demonstrated impaired contextual learning as adults. Thus, the deficits in contextual fear were reversed with dietary choline supplementation. Finally, mice that began nicotine administration and choline supplementation as adults displayed no differences in contextual learning compared to saline and standard diet controls. Taken together, this indicates that choline alone, at the concentration tested, does not enhance hippocampus-dependent learning but will reverse adult deficits in contextual fear learning that result from chronic nicotine administration in adolescence.

One way in which choline may reverse the observed learning deficits is by increasing acetylcholine through enhancing acetylcholine synthesis (Murai et al., 1994). It is possible that chronic adolescent nicotine treatment leads to decreased acetylcholine production. This is indicated by previous research demonstrating chronic adolescent nicotine treatment in rats persistently decreased both ChAt activity and the high-affinity choline transporter (HC-3) expression (Trauth et al., 1999; Trauth et al. 2000). Additional choline supplemented through the diet may increase the rate of synthesis of acetylcholine when it is needed for cognitive processes, such as in contextual fear conditioning. This is

an important consideration since increased transmission of acetylcholine in the hippocampus was observed during both the acquisition and retention of contextual fear learning (Nail-Boucherie et al., 2000). Furthermore, artificially depleting acetylcholine levels as a result of chronic treatment with acetylcholinesterase (AChE) led to working memory deficits, potentially due to the decreased availability of acetylcholine during learning. It was found that chronic, but not acute administration of MKC-231, a choline uptake enhancer, reduced working memory deficits by increasing the synthesis and release of acetylcholine (Murai et al., 1994). In the current study, choline administration following chronic adolescent nicotine treatment reversed the deficits in adult contextual fear learning (hippocampus-dependent) and also enhanced cued fear conditioning in the early adolescent group, irrespective of drug treatment. This indicates that choline supplementation likely enhanced acetylcholine synthesis and availability. Thus, chronic choline supplementation for 30 days may reverse the persistent learning deficit resulting from adolescent nicotine by increasing the availability of choline in order to increase acetylcholine release during contextual fear learning.

It is possible that choline acts through epigenetic mechanisms, altering DNA expression. For example, choline administration during the prenatal and postnatal periods decreased the amount of time to acquire a spatial task in adult rats and also facilitated contextual fear learning in adult rats (Meck et al., 2008; Lamoureux et al., 2008). That is, choline administration in utero facilitated learning and memory in adulthood without the need for choline to be on board during learning tasks. This indicates that choline during development may enhance learning and memory at later time points through long-lasting changes and one possible process is epigenetic changes.

In support of this idea, chronic ethanol exposure during the perinatal period in rats resulted in DNA hypermethylation in the hippocampus and PFC that was reversed with choline supplementation, indicating choline can reverse epigenetic changes resulting from drug exposure during critical periods of development (Otero et al., 2012). Epigenetic modulation by choline supplementation may underlie the reversal of deficits caused by adolescent nicotine exposure as clinical studies have shown differences in DNA methylation in adolescents whose mothers smoked during pregnancy (Lee et al., 2015). Other research has supported the notion that choline supplementation alleviates cognitive deficits by changing gene expression patterns in the hippocampus and cortex (Niculesu et al, 2006; Davison et al., 2009; Otero et al., 2012). Thus, if nicotine administration during adolescence causes changes in gene expression that underlie the learning deficit, it is possible that choline administration helps to reverse these genetic changes leading to improved learning performance.

In addition to increased availability for the synthesis of choline, and epigenetic modifications, it is possible that choline supplementation helps by activating α7 receptors as choline is a selective α7 agonist (Alkondon et al., 1997; Mike et al., 2000). This is important considering the α7 receptor has been implicated as a therapeutic target in neurodegenerative diseases as activation of α7 receptors alleviated associated cognitive deficits (Leiser et al, 2007; Thomsen et al., 2010). Thus, administration of choline may override the behavioral deficits of chronic nicotine exposure by acting through the α7 receptor. For example, choline binding to α7 can evoke the release of neurotransmitters, including ACh and glutamate release in the hippocampus (Cheng & Yakel, 2015; Alkondon et al., 1997). Thus, the chronic administration of choline may ameliorate

cholinergic deficits by increasing the transmission of neurotransmitters crucial for working memory (Yuen et al., 2009; Gold, 2003). Additionally, it has been shown that the α7 nicotinic receptor plays a large role in synaptogenesis and axonogenesis during early adolescence and these processes can be disrupted with the addition of nicotine (Slotkin et al., 2004; Chan & Quik, 1993). Furthermore, since choline administration started after nicotine administration, it is possible that if nicotine disrupted these processes during adolescence the administration of choline worked to reverse these alterations to support learning and memory in adulthood. Choline may reverse these deficits by promoting these processes through the activation of α7 nAChRs. In sum, choline, acting as a selective α7 agonist, may decrease deficits in adult contextual fear learning following adolescent nicotine exposure.

Changes in neurogenesis following chronic choline administration may be another possible mechanism to explain the role choline plays in reversing deficits in adult contextual fear learning. Neurogenesis is a crucial component to contextual fear learning and previous work has demonstrated ablation of neurogenesis attenuated contextual fear learning in adult mice (Saxe et al., 2006). Furthermore, adult administration of nicotine decreased neurogenesis in the hippocampus (Abrou et al., 2002; Campbell et al., 2010). Thus, chronic nicotine administration during adolescence may impair adult contextual fear learning by decreasing cell proliferation and survival in the hippocampus and choline supplementation may reverse these effects. Improved cognitive performance may be due to choline increasing hippocampal neurogenesis, as previous work has shown female rats exposed to choline supplementation in utero demonstrated increases in neurogenesis in adulthood (Glenn et al., 2008). However, it is unclear if chronic administration of

nicotine during adolescence leads to changes in adult neurogenesis. Pilot data collected with Dr. Ellen Unterwuld's lab suggested there was no baseline changes in adult neurogenesis following chronic adolescent nicotine exposure using the same dosing regimen and protracted abstinence model employed by the current study. It may be the case that while the dose and duration used in the current studies led to deficits in adult contextual fear, the same treatment protocol may be insufficient to cause long-term decreases in baseline adult neurogenesis. Furthermore, chronic adolescent nicotine treatment may attenuate learning-dependent neurogenesis while not affecting baseline measures of cell survival and proliferation. The ability for nicotine to decrease neurogenesis is an important consideration because previous research demonstrated α7 receptors are crucial to regulating adult neurogenesis (Campbell et al., 2010) and, as mentioned previously, choline is a selective α7 agonist. Thus, choline may act by promoting neurogenesis during learning trials to reverse the deficits in adult contextual fear after chronic adolescent nicotine exposure. In conclusion, it is unknown if choline administration during the adolescent period increases neurogenesis and whether such an increase would improve adult learning deficits arising from adolescent nicotine abuse and this may be a direction for future studies.

The current experiments are also in agreement with other research showing that choline supplementation during critical periods of development can attenuate cognitive deficits in animal models of fetal alcohol syndrome. This further supports the idea that choline supplementation can reverse cognitive deficits that arise from drug exposure during critical periods of development. For example, it was found that supplementing choline through the diet reversed many cognitive deficits associated with fetal alcohol

syndrome (Velazquez et al., 2013; Wong-Goodrich et al., 2008; Thomas et al., 2000). Specifically, exposure to alcohol during the perinatal period caused deficits in a visual discrimination task at P45 and these deficits were reversed with choline administration from P2-P20 (Thomas et al., 2000). Additionally, choline supplementation during both periadolescence and adolescence attenuated deficits in the Morris Water Maze, a hippocampus-dependent spatial task (Logue et al., 1997), associated with ethanol exposure during the prenatal period (Ryan et al., 2008). In rats exposed to alcohol during gestation, choline supplementation reversed deficits in trace conditioning (hippocampus-dependent; Burman et al., 2006) but not in standard cued conditioning, suggesting that the reversal effects of choline supplementation are limited to hippocampus-dependent learning (Thomas & Tran, 2012). Our results are in agreement with this as choline administration did not enhance cued learning in late adolescent or adult animals when tested in adulthood. In contrast, adult mice treated with nicotine and standard chow had increased cued learning relative to mice that received saline and standard chow. However, this finding is difficult to interpret as results from aim 1 did not show similar changes in cued conditioning following chronic nicotine treatment during adulthood. Further, other researchers have suggested that the benefits of choline administration are limited to early periods of development and this may explain why early adolescent mice treated with choline produced better adult cued learning relative to standard diet controls (Glenn, Adams, and McClurg, 2012). Thus, while choline administration reversed the adult deficits in contextual fear learning associated with adolescent nicotine exposure, the therapeutic benefits of choline administration may be limited to critical periods of brain growth.

In conclusion, chronic adolescent nicotine treatment results in deficits in contextual fear learning in adulthood that are reversed with chronic dietary administration of choline. Since chronic nicotine treatment leads to dysregulations in the acetylcholinergic system (Trauth et al., 2000), the administration of choline could reverse deficits by providing a boost in the production of acetylcholine, thereby facilitating contextual fear memories. It is also possible that the reversal of learning deficits caused by nicotine during adolescence is related to epigenetic modifications. Further, choline is a selective α7 agonist, and other α7 agonists have been shown to improve deficits associated with neurodegenerative diseases, and therefore choline supplementation may negate the cognitive deficits through the effects of the α7 nAChR (Alkondon et al., 1997; Mike et al., 2000). This is in line with previous work showing choline administration during periods of development can reverse deficits associated with fetal alcohol exposure (Velazquez et al., 2013; Wong-Goodrich et al., 2008; Thomas et al., 2000). Future studies need to examine which mechanism contributes to the amelioration effects of choline or if it is an interaction between multiple mechanisms.

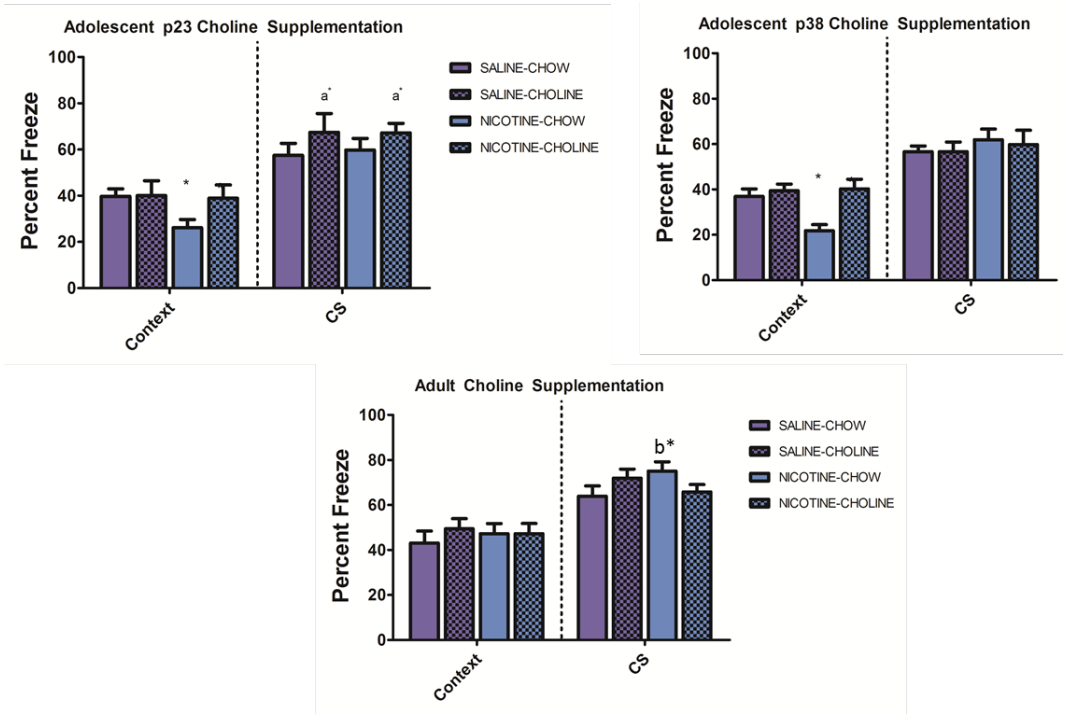


Figure 6: Choline supplementation reverses contextual fear deficits. Choline (9g/kg) supplemented through the diet reversed the deficits in adult contextual fear caused by adolescent nicotine treatment. There were no effects on adult choline supplementation on contextual learning later in adulthood. Bars represent mean percent freezing scores graphed with standard error of the mean (SEM). * $p < 0.05$ for simple main effects comparing diet effect within nicotine condition; a* = $p < 0.05$, main effect of diet condition , b* = p <0 .05 for NIC-CHOW compared to SAL-CHOW conditions in adulthood.

CHAPTER 4

EXAMINING LONG-TERM AFFECTIVE CHANGES FOLLOWING CHRONIC NICOTINE IN EARLY ADOLESCENCE, LATE ADOLESCENCE, AND ADULTHOOD

Rationale

It is well known adolescent nicotine use increases the risk for affective disorders later in life. Several studies have observed a relationship between adolescent smoking and depressive symptoms in adulthood. For example, in a longitudinal study tracking adolescents and young adults over a period of 13 years, it was found that adolescent smoking was predictive of depressive symptoms in adulthood (Brook et al., 2004). Even more compelling evidence comes from a longitudinal study of adolescents that showed no differences in depressive symptoms at baseline, but those that were identified as established smokers, characterized by having smoked at least 100 cigarettes in their lifetime or had smoked within the last 30 days, had twice the risk of developing depressive symptoms when assessed four years later (Choi et al., 1997). Thus, nicotine abuse during adolescence may lead to a negative affective state, as well as cognitive impairments later in life, further increasing the probability of continued use in adulthood. Additionally, stress experienced during the adolescent period is positively correlated with the risk of developing anxiety disorders and depression later in life (Compas, Orosan, & Grant, 1993). Elevated anxiety increases the risk for nicotine dependence and smokers often cite alleviating anxiety as a reason to continue smoking (Bresalau, Kilbey, & Andreski, 1991; McCabe et al., 2004; Watson et al., 2012). Thus, it is unclear if the

interaction between adolescent stress and adolescent nicotine causes persistent changes in depressive-like and anxiety-like behavior.

The interaction of adolescent stress and adolescent nicotine causes observable deficits in learning and it is possible that this interaction would cause changes in depression and anxiety as well, as mood disorders are often associated with impaired cognition and deficits in working memory (Harvey, 2007; Lupien et al., 1999; Austin et al., 2001). As we know that 1) reductions in hippocampal volume are associated with clinical depression (Bremner et al., 2000), 2) early life stress causes a reduction in adult hippocampal neurogenesis that corresponds to increases in depressive behaviors (Lemaire et al., 2000; Bahra et al., 2011), and 3) adolescent nicotine and adolescent stress cause impairments in hippocampal-dependent learning, while sparing types of learning that are not critically dependent on the hippocampus, (Portugal et al., 2012), it is possible that adolescent nicotine exposure increases adult depressive-like behaviors following chronic adolescent nicotine. Thus, these experiments compared the effects of adolescent nicotine exposure on adult depression and anxiety with the prediction that depression will be specifically disrupted by adolescent nicotine exposure.

Methods

Subjects and Experimental Conditions

C57BL/6J male mice obtained from Jackson Laboratories (Bar Harbor, ME) at either post-natal day (p) p16 (early adolescent), p31 (late adolescent), or p47 (adulthood). Early adolescent mice were shipped with dams and weaned at p21. Each age had two treatment conditions consisting of chronic nicotine (n=7-8) or chronic saline

administration (n=7-8). Mice were housed in groups of 4 and given ad libitum access to food and water.

Drugs

One week following arrival at our animal facility mice were subcutaneously implanted with mini osmotic pumps to deliver nicotine (Sigma, St. Loius. MO; free base, 12.6mg/kg/day) at p23, p38, and p54, or underwent sham surgeries at the ages the same as procedures outlined previously. Nicotine or saline was delivered continuously for 12 days at which time the mini pumps were removed. Mice then went through a 30 day drug wash out period before beginning a three day behavioral battery (see figure 7).

Depression and Anxiety Assay.

All mice underwent three days of behavioral testing in various procedures designed to test depression-like and anxiety-like behavior. Specific procedures for each

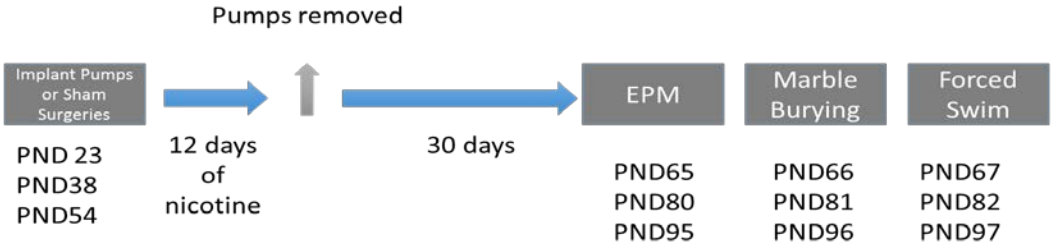


Figure 7: Depiction of ages at experimental time points used for the depression and anxiety battery of tests.

task are outlined below. On the first day, mice were placed in an elevated plus maze (EPM) apparatus for 5 minutes to assess anxiety-like behaviors. On the second day, mice were observed for anxiety and obsessive like behaviors in a marble burying task. Finally,

on the last day of testing, mice were assessed in the forced swim test (FST) to measure depression-like behavior.

Elevated Plus Maze. Thirty days following cessation of chronic administration of nicotine, mice were evaluated for changes in anxiety-like behavior using the elevated plus maze. The maze consisted of a wooden base (62.6cm) elevating a plexiglass platform that consists of two open arms (7.6 × 30.6 cm) and two closed arms (7.6 × 30.6 × 15.5cm) and an open area in the center of the maze. Mice were placed in the center area of the maze facing a closed arm and recorded with a video camera mounted overhead. Videos were scored for time in open arms, time in closed arms, and time spent in the center for the 5 minute test duration (Kenney, Portugal & Gould, 2012).

Marble Burying. In order to assess anxiety-like and compulsive behaviors, a marble burying task was used thirty-one days after drug administration. Mice were placed in cage (30 × 20cm) with 5cm of bedding covering the bottom. Twenty marbles lined the perimeter, approximately 4cm apart. Mice were left undisturbed for the 30 minute test duration and were recorded with a video recorder for later analysis of behavior. Number of marbles buried as well as time spent digging were measured. Marbles were considered buried if they were covered greater than 2/3 in the bedding (Deacon, 2006).

Forced Swim. Thirty-two days after administration of drug, mice were evaluated for changes in depressive like behaviors. Forced swim consists of placing a mouse in a clear cylinder (20cm × 40cm) filled with 15 cm of water (25C) and recording the subject for a period of 6 minutes. Similar time points have been used in published studies (reviewed in Can et al., 2012). Mice were continuously monitored throughout and removed from the cylinder early if struggling.

Statistical analysis

In order to assess age- and drug-dependent effects a separate 2 [drug: saline versus nicotine] × 3 [age: p23, p38, p54] ANOVA was run for the dependent measures on each behavioral task. Significant main effects were followed up with post-hoc tests and significant interactions were followed up with simple main effects analysis. Finally, correlations between the dependent measures on all tests were calculated using Pearson's r to determine if there were significant relationships between depression and anxiety measures.

Results

EPM

When assessing anxiety-like behavior in the EPM task, there was a significant main effect of treatment condition on time spent in open arms ($F(1,38) = 18.25$, $p < .001$) and time spent in the closed ($F(1,38) = 10.33$, $p < 0.01$) (see figure 7). Mice that received nicotine spent less time in the open arms and more time in the closed arms than mice that received saline when tested 30 days later. Tukey's post-hoc tests indicate that there was a significant difference between adult saline and adult nicotine mice ($p < .05$). There was a significant effect of age in time in the center ($F(2,38) = 3.36$, $p < 0.05$) where early adolescent mice spent significantly more time in the center than adult mice ($p < 0.05$). Thus, chronic nicotine treatment persistently increases anxiety-like behavior in adult mice treated with nicotine during adulthood but tested 30 days later.

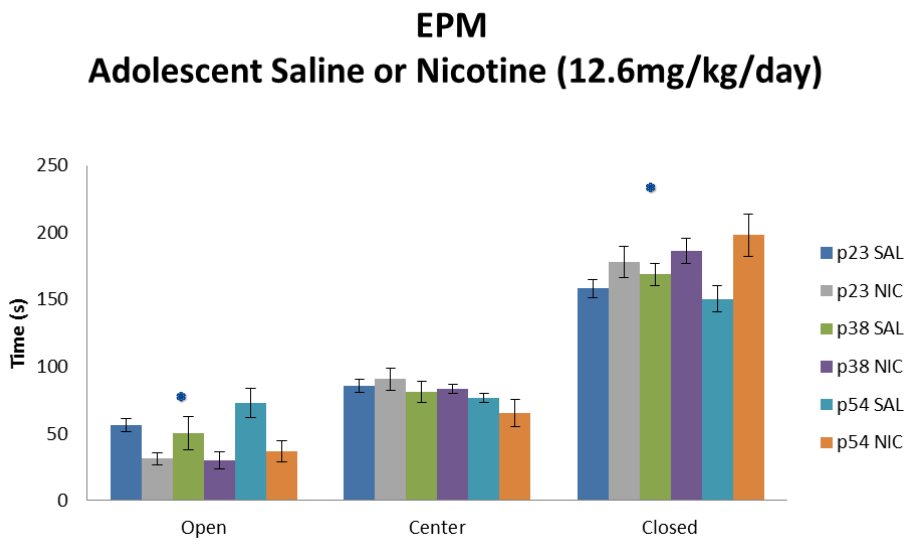


Figure 8: Chronic nicotine leads to enduring effects on anxiety. This graph depicts time spent in the open, center, and closed areas of the EPM. Chronic nicotine administration leads to persistent increases in anxiety and this effect is most robust following adult administration. Bars represent mean time in seconds. * $p < 0.05$ compared to saline age-matched controls

Marble Burying

There were no drug effects when assessing obsessive- and anxiety-like behavior in the marble burying task. Mice treated chronically with saline or nicotine buried the same amount of marbles. There was a significant main effect of age, $F(2,40) = 5.90$, $p < .01$. Tukey post-hoc tests indicate that adult mice exposed to nicotine in early adolescence buried less marbles than late adolescent exposed group ($p = 0.007$) and there was a trend in the same direction compared to the adult group ($p = 0.07$). This indicates that early adolescent mice may be more sensitive to methodological stressors than other age groups as all experimental groups were treated in the same manner. There we no significant interactions between age and drug condition.

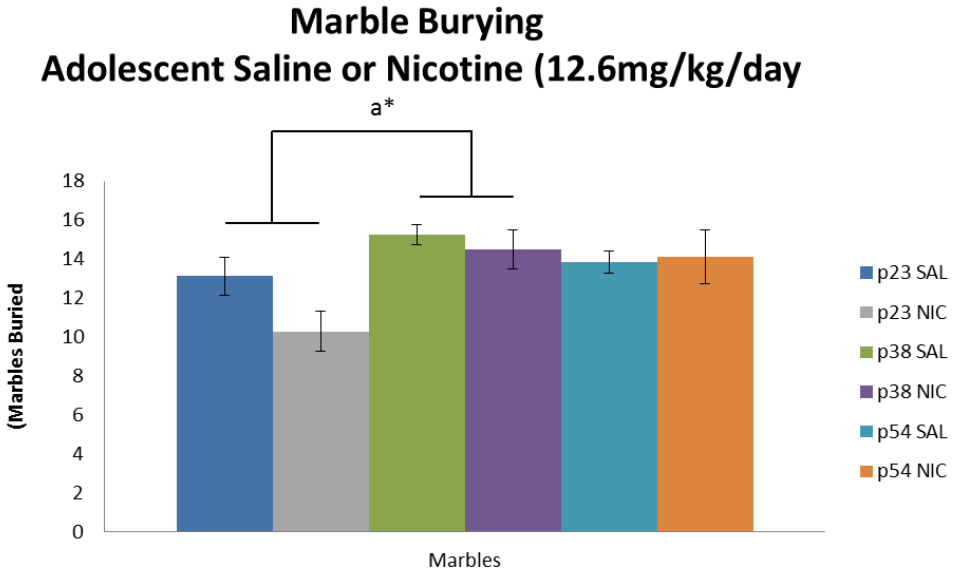
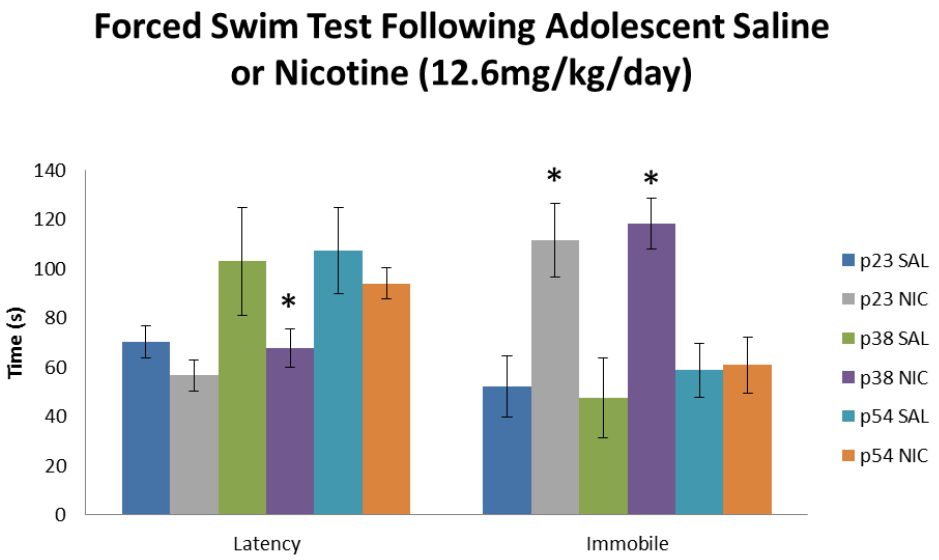


Figure 9: No changes in marbles buried following chronic nicotine. This graph depicts total marbles buried after 30 minutes. Chronic nicotine administration had no effects on marbles buried but early adolescent mice buried less marbles than other ages. a* $p < 0.05$ indicates significant difference between the early adolescent and late adolescent in total marbles buried regardless of drug treatment.

Forced Swim Test

Early adolescent and late adolescent mice treated with nicotine had decreased latency to immobility and increased time spent immobile as adults, indicating increased depressive-like behaviors (see figure 8). There were significant main effects of age ($F(2,46) = 5.26$, $p < .01$) and treatment condition ($F(1,46) = 5.16$, $p < .05$) in latency to immobility in the forced swim test. Early adolescent had shorter times to immobility than adult mice ($p < .05$) and overall, mice treated with nicotine had shorter times to

immobility than saline treated mice ($p < .05$). When measuring immobility, there was a significant main effect of treatment condition where mice that received nicotine had longer bouts of immobility than saline mice, $F(1,46) = 20.02$, $p < .01$). There was also a significant interaction between age and drug ($F(2,46) = 4.16$, $p < .05$). Simple main effects indicates that for both early adolescence ($F(1,27) = 11.44$, $p < .01$) and late adolescence ($F(1,27) = 17.23$, $p < .001$), nicotine-treated mice had significantly longer bouts of immobility than saline controls when tested during adulthood. There was no significant difference in adult mice between saline and nicotine groups. Consequently, persistent increases in depression-like behaviors following chronic nicotine



administration were age-dependent.

Figure 10: Chronic nicotine during adolescence increases depressive-like behaviors. Increased depressive-like behaviors are seen after adolescent nicotine. Decreased latency to immobility and increased time spent immobile are seen only inadult mice that received nicotine during adolescence when compared to their saline controls. Means are expressed as time in seconds. * $p < 0.05$ compared to saline age-matched controls.

Correlations

In order to determine the relationship between anxiety and depression measures, dependent measures for all behaviors were correlated. It was found that time spent in the open arm and time spent in the closed arm were negatively correlated ($r = -.81$, $p < .01$). Also, time spent in the open area of the EPM was negatively correlated to time spent in the center ($r = -.64$, $p < .01$). Additionally, time spent immobile was negatively correlated to latency to immobility ($r = .61$, $p < .01$), indicating shorter times to immobility were related to the time spent. Finally, latency to immobility and number of marbles buried were positively correlated ($r = .30$, $p < .05$). This indicates that depressive-like behaviors were positively correlated to specific types of anxiety measures. There were no significant correlations between EPM measures and marbles buried, indicating these two measures of anxiety-like behaviors may be probing different underpinnings of anxiety. Altogether, the results demonstrate there is a significant relationship between dependent measures on the EPM, a strong relationship between depressive-like behaviors in the forced swim, and a moderate relationship between specific types of anxiety measures and depression measures.

Discussion

The objective of these experiments was to determine if chronic nicotine beginning at different developmental time points caused long-term affective changes measured by a three day behavioral assay that included the elevated plus maze, marble burying, and forced swim. The major findings from this study suggest that chronic nicotine administration during adulthood leads to persistently increased anxiety when measured using the EPM during later time points of adulthood. In the marble burying task there were no effects of drug treatment indicating that chronic nicotine administration may only increase anxiety in some situations but not all. Also important was that mice in the early adolescent cohort buried significantly less marbles than other ages regardless of treatment condition. This indicates that procedural stress, such as that from transportation, surgery, or handling, may have age-dependent effects on behavioral outcomes since all mice in the study were subjected to the same methodologies. Finally, chronic nicotine administration during early adolescence or late adolescence led to enduring depressive-like behaviors in adulthood. Both saline and nicotine treated adult mice, on the other hand, had similar levels of depressive-like behaviors. In sum, chronic nicotine treatment leads to long-term affective changes in an age-dependent and task-dependent manner. It appears that chronic nicotine administration in adolescence leads to significant increases in immobility in the forced swim task in adulthood, chronic nicotine administration in adulthood has the most profound effects on anxiety measures, and experiment related stress during early adolescence may alter behaviors independently of drug treatment.

The most interesting finding from this study is that chronic nicotine administration during early and late adolescence increased depressive-like behaviors in adulthood. This finding is in line with previous work demonstrating that daily injections of nicotine during adolescence in rats resulted in decreased sucrose preference and increase immobility in the forced swim in adulthood. Like our study, adult rats given daily injections and tested later in adulthood do not show the same propensity for depressive behaviors (Inguiez et al., 2009). Researchers also found that administration of fluoxetine, a selective serotonin reuptake inhibitor and commonly prescribed antidepressant, reversed the depression-like state in adulthood in mice that received nicotine in adolescence. Additionally, acute administration of nicotine at the time of the forced swim test also reversed the depressive-like behaviors (Inguiez, 2009). Since the hippocampus and the serotonergic system reaches maturity (reviewed by Abreu-Villica et al., 2011) by the time the adult mice in the proposed study receive nicotine it is unsurprising that there were no increases in immobility. Taken together this indicates that adolescent nicotine potentially causes dysregulations of the serotonergic system that persists well into adulthood.

Results from the current study suggests that chronic nicotine administration leads to increased anxiety in adult mice, measured by decreased time spent in open arms and increased time in the closed in the elevated plus maze. Elevated anxiety increases the risk for nicotine dependence and smokers often cite alleviating anxiety as a reason to continue smoking (Bresalau, Kilbey, & Andreski, 1991; McCabe et al.,2004; Watson et al., 2012). Studies examining the effects of acute and chronic nicotine in adult animals on anxiety-like behaviors have reported inconsistent findings. Some studies report that nicotine is

anxiolytic while others indicate nicotine is anxiogenic. For example, in one study, Brioni et al. (1993) reported that low doses of nicotine increased time spent in the open arms of the EPM in adult male CD-1 mice. In another study, acute administration of nicotine resulted in anxiogenic effects in the EPM at 5 minutes and 30 minutes afterwards (Biala & Budzynska, 2006). These discrepant findings, that nicotine can cause both an anxyiolitic and an anxiogenic effect are consistent with human literature (Brown et al., 2001). Despite the inconsistencies in anxiety-like behaviors following acute or chronic administration of nicotine, most studies that examine anxiety-like behavior following withdrawal from chronic nicotine report that withdrawal from chronic nicotine is anxiogenic (Irvine, Cheeta, & File, 2001; Damaj, Kao, & Martin, 2003l Jonkman et al., 2005; O'Dell et al., 2007; O'Dell et al., 2004; Wilmouth & Spear, 2006). Studies that have examined the effects of nicotine withdrawal on EPM behaviors have indicated that adult mice withdrawn from chronic nicotine spend less time in the open arms than saline controls (Irvine, Cheeta, & File, 2001; Damaj, Kao & Martin, 2003). However, it is important to note that the current model used mice that were chronically administered nicotine and subjected to a 30 day drug wash out period, also known as a protracted abstinence.

Like adults, adolescent nicotine exposure can result in anxiogenic or anxyiolitic effects after acute administration. For example, Kupfershmidt et al. (2010) showed that mid-adolescent Long-Evan rats (P33-37) had less time spent in the open arms of the EPM, compared to adult animals (P65-69) after an acute dose of nicotine. Further, Elliot and colleagues (2004) demonstrated that repeated injections of nicotine in adolescent Sprague-Dawley rats (P25-30) and adult rats (P55-60) resulted in an age- and sex-

dependent effect on anxiety. Adolescent males showed anxiolytic behaviors, with increased percentages of time in the open arms, whereas adolescent females, and adult males and females, showed anxiogenic effects. However, research has consistently shown adolescent mice undergoing nicotine withdrawal show less somatic signs of withdrawal, including no changes in anxiety-like behaviors (Wilmouth & Spear, 2006; O'Dell et al, 2004). Fewer studies have examined the long-term effects of adolescent nicotine administration on adult anxiety. Smith et al. (2006) examined the effect of chronic nicotine administration during adolescence and found increases in anxiety when rats were tested an open field assay in adulthood. The increases in anxiety in adult rats were due to nicotine exposure during adolescence, as nicotine was not administered during testing in adulthood. On the other hand, Abreau-Villica et al. (2008) found no differences in anxiety in adulthood following saline or nicotine treatment in adolescence, which is in agreement with the current study. However, it is important to note that the nicotine dose used in 2008 study does not raise cotinine levels to the same level (Klein et al., 2004) as those in previous studies that have found enduring effects in anxiety following chronic nicotine administration (Smith et al., 2006). Thus, the differences between the current study and the Smith et al. (2006) study may be due to the higher dose of nicotine administered during adolescence and future work should investigate whether enduring anxiety following adolescent nicotine exposure is dependent on dose of nicotine.

One advantage of this study was the use of a battery of tests which included a secondary measure of anxiety and compulsive behaviors through the use of marble burying. Marble burying takes advantage of the natural inclination to bury objects in their den and is used in research to measure changes in anxiety or obsessive behaviors

(Deacon, 2006). Specifically, higher numbers of marbles buried typically indicates higher levels of anxiety. Some researchers have proposed that the marble burying task is better at identifying anxiolytic agents than anxiogenic (Njung'e & Handley, 1991) so the lack of an effect between drug groups is unsurprising given the results that nicotine increased anxiety measures in only the adult cohort in the EPM task. Interestingly, previous research has shown marble burying is not correlated to other commonly used assays to measure anxiety (Thomas et al., 2009). Yet, previous work has indicated that marble burying behaviors correlate to immobility in the forced swim, which results from the current study support (Kobayashi et al., 2008). Interestingly, Kobayashi's study pointed out that antidepressants that suppressed immobility behaviors in the forced swim were correlated with increases in extracellular dopamine release whereas antidepressants that suppressed marble burying correlated with increases in extra cellular serotonin release. Thus, while these two behaviors were related they were driven by two different systems which may expound why chronic nicotine administration during adolescence, but not adulthood, led to increased depressive-like behaviors in the forced swim while not increasing measures of anxiety. It is also important to note that mice treated during early adolescence (p23) had lower numbers of marbles buried compared to all other ages, regardless of drug treatment. This may indicate that early adolescence is a critical period for long-term alterations in anxiety-like behavior resulting from methodological stressors such as surgeries and handling. In sum, the results from marble burying coupled with the EPM indicate that chronic nicotine does not lead to persistent increases in anxiety in adulthood and this is independent of increases in depression-like behavior.

In conclusion, chronic nicotine administration during early adolescence and late adolescence significantly increases depressive-like behaviors in adulthood. This finding is also supported by clinical literature indicating adolescent smoking is a risk factor in the development of depression later in life. Furthermore, other studies showing increased depressive behaviors following chronic nicotine during adolescence successfully reversed the depressive behaviors with the addition of fluoxetine, suggesting adolescent nicotine leads to enduring alterations in the serotonergic system. There was also a persistent increase in anxiety following chronic nicotine treatment but only in adult mice that received nicotine during adulthood. This is troublesome as anxiety reduction is often cited as a reason to continue smoking (Brown et al., 2001). However, when analyzing marble burying there were no increases in anxiety in any of the age groups as a function of drug treatment. Thus, increases in anxiety following chronic nicotine administration may be task dependent. Finally, taken together with the findings that adolescent stress and adolescent nicotine independently alter cell structure and function in the hippocampus, the interaction of stress and nicotine during adolescence created deficits in hippocampus-dependent learning, and alterations in the hippocampus are correlated with depression, the findings from this study further supports that adolescence is a vulnerable time to long-term changes in hippocampal systems that are involved in depression and cognition during adolescence but this was not directly tested. These effects could also explain why smoking initiation occurs at younger ages, as nicotine exposure during early adolescence results in anxiety reduction (File, Fluck, Leahy, 2001) but could foster dependence later in life by impairing learning and increasing depressive symptoms. In order to best treat the persistent cognitive deficits that arise following adolescent stress

and nicotine it is also imperative to treat and monitor the increased risk for developing depression.

CHAPTER 5

CONCLUSIONS

While smoking rates have steadily declined in the United States in the last 4 decades, it seems to have leveled off at around 18.1% of the general population (CDC, 2014). Changes in affect and cognition following withdrawal from nicotine are often cited as reasons for relapse, but the issue is more complex because of the neurobiological adaptations occurring in response to chronic nicotine treatment. Continued prevalence rates of smoking are also maintained by those initiating tobacco use in adolescence, as younger initiation of smoking leads to a greater risk of dependence in adulthood. Adolescence represents a unique time for enduring alterations caused by nicotine that can impact adult behaviors. Thus, the persistent cognitive and affective impairments resulting from chronic nicotine exposure in adolescence can promote continued use of nicotine. Studies conducted here also support the notion that these deficits are not solely a result of nicotine but an interactive effect between nicotine and stress during adolescence.

Activation of the stress system ultimately leads to an elevation in circulating glucocorticoids; prolonged elevations of glucocorticoids can lead to a multitude of negative consequences such as increases in anxiety and impairments of learning and memory. Nicotine can activate the stress response and acute nicotine can also result in a rise in corticosterone. Regardless, alleviation of stress is often given as a reason to engage in smoking in clinical populations, and populations that experience a higher degree of daily stress have higher smoking prevalence rates (Voglie, 2005). This is problematic because exposure to either nicotine or stress results in dysregulations of the stress system and systems that support learning and memory. During adolescence,

activation of the stress system can lead to long-lasting changes in brain structures that support normal stress responses and cognitive functions, much like how nicotine can induce long-term changes. Results from the few studies that have examined stress during adolescence reveal that there are enduring effects on responses to nicotine (McCormick et al., 2004), which may explain why adolescents, a group that consistently reports higher levels of perceived stress (Finkelstein et al., 2006; Siqueria et al., 2000), are both more prone to initiate smoking and face more intense tobacco dependency later in life (Chen & Millar, 1998). The studies conducted here indicate that stress, either through shipping stress or a combination of restraint and social instability stress in adolescence prior to administration of nicotine drives the resulting in deficits in hippocampus-dependent learning and depression. Furthermore, the long-term impairments in hippocampus-dependent learning occur despite no differences in baseline alterations in the stress system throughout adolescence and adulthood. Taken together this indicates that stress and nicotine interact in such a way that may cause epigenetic changes in areas outside of the stress system that lead to learning impairments.

In support of the possible epigenetic mechanism of adolescent stress and adolescent nicotine, the second aim of these experiments was to test if choline supplementation, a necessary precursor to acetylcholine and a known modulator of DNA methylation (Davison et al., 2009), would reverse the observed learning deficits. While prior research suggested that chronic adolescent nicotine treatment leads to dysregulations in the cholinergic system of the adult hippocampus (Trauth et al., 1999; Slotkin et al., 2000), and other experiments have shown deficits in adult hippocampus-dependent learning following chronic nicotine administration (Spaeth et al., 2010;

Portugal et al., 2012), these experiments offer a some mechanistic explanations for the observed deficits seen in my study. In other words, nicotine during adolescence likely creates inefficient cholinergic signaling which leads to deficits in contextual fear learning and dietary choline supplementation reverses these deficits through providing support to the cholinergic system. Furthermore, it is possible that nicotine and stress during adolescence causes differences in DNA methylation that were reversed with dietary choline administration, which would help ameliorate the learning deficits. Future studies should consider the role of epigenetic modulations and ways to reverse irregular patterns of methylation in the long-term deficits in adolescent nicotine abuse.

The final aim of these studies was to determine if adolescent nicotine treatment resulted in alterations in depression and anxiety in adulthood. Anxiety reduction and mood enhancement are commonly cited for reasons to continue smoking in clinical literature (Carmody, 1989; Brandon and Barker, 1991). It was found that chronic nicotine during early and late adolescence, but not adulthood, increased depressive measures in adulthood. However, there were no differences in anxiety following adolescent nicotine but a modest increase in anxiety following adult nicotine treatment. It is important to point out though that the increase in anxiety resulting from adult chronic nicotine was only observed in the EPM and not marble burying, suggesting this may be a modest effect. Nonetheless, since adolescent chronic nicotine administration did increase depressive-like behaviors, this helps to understand why adolescent smokers have more severe dependence later in life. This is troubling since the risk for depression is greater in adolescent smokers, cognitive impairments are seen after adolescent smoking, and depression and anxiety are often associated with cognitive impairments. Results from

these studies indicate changes in affect may be correlated with the learning deficits and efforts to minimize the impacts of adolescent nicotine abuse should address both cognitive and affective impairments.

Currently, the CDC has reported that the most effective forms of reducing adolescent nicotine use is by limiting marketing by tobacco companies along with aggressive counter advertising campaigns to encourage staying tobacco free. This has led to a marked reduction in adolescent smoking (CDC, 2014). However, there are still many individuals who will abuse nicotine during adolescence, through conventional cigarettes or through electronic cigarettes, and may find themselves dependent on nicotine in adulthood. As these studies and countless others have indicated, these individuals are more prone to cognitive and affective impairments that will, unfortunately, increase the risk for continued use. Results from the current studies show the importance of identifying other, sometimes overlooked, variables, such as adolescent stress, that mediate adolescent nicotine abuse and the resulting impairments. Characterizing these deficits at the biological and behavioral levels allows for the development of effective treatments, such as the reversal of learning impairments through dietary choline supplementation. Understanding the affective states that may also contribute to continued nicotine abuse or augment the resulting cognitive deficits also provides useful information to break the cycle of nicotine addiction that still plagues 18.1% of the American population. In order to continue the decline of smoking prevalence that began in the 1970s and stalled in the 2010s it is imperative to identify, address, and reverse the consequences of adolescent nicotine use and abuse.

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