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THE IMPACT OF ADOLESCENT SOCIAL EXPERIENCES ON ADULT
ADRENOCORTICAL ACTIVITY, AFFECT-RELATED BEHAVIOR, AND
NICOTINE RESPONSES

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by

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ABSTRACT

A variety of stress-related psychopathologies including affective disorders such as anxiety, depression, and nicotine use, emerge during adolescence. Development of comorbid affective disorders and nicotine use has been attributed to hypothalamic-pituitary-adrenal axis (HPA) dysregulation associated with chronic stress during adolescent development. However, few studies have simultaneously investigated adolescent stress effects on affective behavior, HPA activity, and nicotine responses in animal models. Rodent models of chronic social stress are ethologically relevant models that may provide valuable insight into mechanisms that underlie adolescent affective disorders and nicotine use comorbidity. However, the results of these studies are inconsistent as they are highly dependent on individual differences in stress susceptibility.

In the current dissertation, I have shown that baseline individual differences in exploratory behavior (i.e. temperament) in outbred rats modulates the impact of adolescent social experience on adult HPA activity (Chapter 2). These findings suggest that temperament is one factor that contributes to inconsistent findings in adolescent stress literature. Additional sources of variability in the literature include genetic variation among outbred rodents and sex differences in stress-susceptibility. For the next study (Chapter 3), I developed a novel adolescent social stress protocol, referred to as chronic variable social stress (CVSS), to control for these potential sources of variability by exposing inbred male and female mice to multiple temperament- and sex-specific stressors. CVSS included repeated cycles of social isolation and housing with novel
social partners. We found that adolescent CVSS increased adult anxiety/depression-related behavior and blunted HPA activity. In the last study (Chapter 4), I replicated and extended these previous findings by simultaneously investigating the impact of adolescent CVSS on anxiety/depression-related behavior, HPA activity, and nicotine responses. I found that male inbred (BALB/cJ) mice exposed to adolescent CVSS exhibited reduced exploratory behavior and altered behavioral and physiological responses to nicotine. However, there was little relationship between anxiety/depression-related behavior and nicotine responses within individuals.

The studies presented herein may help clarify the factors that contribute to inconsistent findings across rodent adolescent social stress models. Furthermore, identification of the factors that promote maladaptive health trajectories (i.e., development of affective disorders and nicotine use) during adolescence can inform future studies on the biological and environmental determinants of adolescent development.
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Preface

Chapters 2-4 of this thesis were published or submitted to peer-reviewed journals for publication.

**Chapter 2:** This chapter is presented as previously published in the journal article: Caruso, M.J., McClintock, M.K., & Cavigelli, S.A. (2014). Temperament moderates the influence of periadolescent social experience on behavior and adrenocortical activity in adult male rats. *Hormones and Behavior, 66,* 517-524. The experiment was designed and by SAC and MSM. Data collection was performed by SAC. MJC analyzed the data, interpreted the results and drafted the manuscript. SAC critically reviewed the manuscript. All co-authors approved the manuscript for submission.

**Chapter 3:** This chapter is currently *in press* and is presented as previously accepted by the journal *Developmental Psychobiology* with co-authors Dr. Helen M. Kamens, and Dr. Sonia A. Cavigelli. MJC and SAC designed and performed the experiment. MJC analyzed the data, interpreted the results, and drafted the manuscript. SAC and HMK supervised data analysis and edited the manuscript. All co-authors approved the manuscript for submission.

**Chapter 4:** This chapter is currently under review and is presented as previously submitted to the journal *Brain Research Bulletin* with co-authors Dana E. Reiss, Jasmine I. Caulfield, Jacob L. Thomas, Allison N. Baker, Dr. Sonia A. Cavigelli, and Dr. Helen M. Kamens. MJC, HMK, and SAC designed the experiments. MJC supervised data collection and all co-authors performed the experiments. MJC analyzed the data, interpreted the results, and drafted manuscript. HMK and SAC edited the manuscript. All co-authors approved the manuscript for submission.
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The trajectory of my career in science has been unconventional. In high-school, I had little interest in anything other than skateboarding. During college, I decided that I wanted more for myself. After a survey of general education courses, I took a leap of faith and transferred to the University of Hawaii where I majored in psychology. I had become fascinated with the “inner workings of the mind” (with little understanding of what that truly entailed) and I was going to be a clinical psychologist. I still don’t know what I was thinking back then. Anyone who knows me could attest that I would be a terrible interpersonal counselor. Until my enrollment at the University of Hawaii I had avoided hard sciences by any means necessary. Then I learned about a brain region called the amygdala and my fears of biology transformed, almost instantaneously, into a deep-seated passion for neurobiology. I decided that I was going to become a behavioral neuroscientist. While searching for research opportunities, I met Dr. Lorey Takahashi who invited me into his lab. Soon thereafter I learned how to cannulate rat amygdalae for drug microinjections. It was in Dr. Takahashi’s lab that I was first introduced to the field of “stress neurobiology” while studying the effects of CRH receptor activity on learning and memory. Ten years later stress is still the cornerstone of my ever expanding scientific interests.

Dr. Takahashi was my first mentor. He was, by no means, my last. I owe a tremendous debt of gratitude to my graduate adviser Dr. Sonia Cavigelli. I would like to thank you for taking a chance on me. I did not, however, become a behavioral neuroscientist in your lab. Rather, you exposed me to a new world of collaborative research that eludes such categorization. Your mentorship has molded my transformation from a naïve 1st year doctoral student into a well-rounded scientist and you have fostered my independence and self-competence. Finally, wherever my scientific career takes me, I believe that rodent behavior will always be at the heart of my research. So, I’d also like to thank you for teaching me everything that I know about animal behavior.

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Finally, I would not have made it this far if not for the love and support of my parents, Wendy and Frank Caruso, brothers, and little sister. Mom and Dad, thank you for guiding me through the tough times. I know that I was not the easiest child to raise, but you never turned your backs on me. I hope that I’ve made you proud. It also goes without saying that your financial support during graduate school has been monumental. FYI - I intend to wean myself off your wallets now. I also have to thank my grandparents, Nielda and Sandy Storms. Grandma, I wish you could have seen me complete this academic journey and start my next one. I know how proud you were and I’m sure every one of your 10,000 friends would have heard about it. Grandpa, I’ve been waiting for you to see me become Dr. Caruso for 10 years. Your attendance at my dissertation defense means the world to me and I’m so glad I was able to share this day with you.
Dedication

I dedicate this dissertation to Grandma and Grandpa Storms. They have showed me so much of the world and instilled in me the importance of both education and family. I hope that someday I can fill shoes half as big as yours and inspire greatness in my children and grandchildren as you have in me.
Chapter 1. Adolescent Development and the Influence of Social Experiences on Affective Disorders and Nicotine Use

1.1. Introduction and significance

Affective disorders, such as anxiety and depression, are complex psychiatric disorders that contribute significantly to the global burden of disease (Greenberg, Fournier, Sisitsky, Pike, & Kessler, 2015; Murray & Lopez, 1996; Steel et al., 2014). Roughly 25% of all Americans will experience these disorders during their lifetime with onset typically occurring during adolescence - a developmental period characterized by profound behavioral and neurobiological maturation (Kessler, Berglund, & Demler, 2005; Spear, 2000). Adolescents are also at greater risk to develop long-term nicotine dependence as 90% of adult smokers initiate smoking before age 20 (United States Department of Health, 1994). Human and animal studies suggest that the developing adolescent brain may be particularly susceptible to long-lasting stress effects that can manifest as affective disorders and comorbid nicotine use (Chaby, Cavigelli, Hirrlinger, Caruso, & Braithwaite, 2015; Eiland & Romeo, 2013; Heim & Nemeroff, 2001; Koenen et al., 2005; McCormick & Green, 2013; Richardson, He, Curry, & Merikangas, 2012; Swendsen et al., 2010). While the etiology of affective disorders and comorbid nicotine use is unknown, both have been associated with adolescent stress-induced hypothalamic-pituitary-adrenal (HPA) axis dysregulation (Burke & Miczek, 2014; Forbes et al., 2006; Heim & Nemeroff, 2001; Holliday & Gould, 2016; Klimes-Dougan, Hastings, Granger, Usher, & Zahn-Waxler, 2001; Rao, Hammen, London, & Poland, 2009b). The ability to limit or prevent the effects of adolescent stress on these maladaptive health trajectories
critically depends on knowledge of the biological and environmental determinants that impinge on the development of the HPA axis and brain regions that regulate reward- and affect-related behavior during adolescence.

Maturation of social behavior (i.e. social interactions with peers) is an important component of adolescent development; social interactions are exceptionally salient as adolescents fine-tune complex social skills (McCormick, Hodges, & Simone, 2015; Spear, 2000). Importantly, social stressors are highly potent stress stimuli in a wide variety of species across all stages of development (Blanchard, McKittrick, & Blanchard, 2001). Given the importance of adolescent social behavior and the salience of social stress, even mild social challenges may be particularly stressful at this developmental stage. Preclinical rodent studies further suggest that adolescents are vulnerable to social stressors during development. For example, several social stress procedures, including social isolation, social defeat, and unstable social housing (social instability), have been shown to increase adult anxiety- and depression-like behaviors in rats and mice (Bourke & Neigh, 2011; Hong et al., 2012; Lukkes, Mokin, Scholl, & Forster, 2009; Ros-Simó & Valverde, 2012; Scharf, Sterlemann, Liebl, Müller, & Schmidt, 2013; Schmidt, Scharf, Liebl, et al., 2010; Schmidt, Trümbach, et al., 2010; Vidal et al., 2007). Moreover, rats exposed to adolescent social instability exhibit altered sensitization to nicotine’s locomotor-activating effects (McCormick & Ibrahim, 2007; McCormick, Robarts, Gleason, & Kelsey, 2004). However, the effects of rodent adolescent social stress paradigms are quite variable. Several studies have reported no lasting effects of stress on HPA axis activity or affective and nicotine behaviors (Boleij et al., 2014; Isgor, Kabbaj,
Akil, & Watson, 2004; Lukkes et al., 2009; McCormick & Ibrahim, 2007; McCormick et al., 2004; McCormick, Smith, & Mathews, 2008; Scharf et al., 2013).

Disparate findings may result from significant individual differences in vulnerability to adolescent social stress. However, little is known about the factors that shape individual differences in vulnerability. Adolescent social stress paradigms typically include genetically diverse outbred animals in which individual differences may be a particular confound, few include both sexes, and the influence of stress-related temperament is rarely considered. These methodological factors can contribute to inconsistent findings in the literature (Boleij et al., 2014; Huang et al., 2013; Scharf et al., 2013; Schmidt, Trümbach, et al., 2010).

Ultimately, we aim to clarify factors that contribute to variability in the effects of adolescent social stress. Our secondary aim is to develop a rodent model of adolescent social stress that capitalizes on these sources of variability and investigate the impact of adolescent social stress on adrenocortical activity and comorbid affective disorders and nicotine dependence.

In this chapter, I will review the literature on adolescence as a developmental period characterized by socioemotional and neurobiological changes that are similar across humans and rodents. I will also present studies on the effects of adolescent stress on affective behaviors (e.g., anxiety- and depression-like behavior) and nicotine responses including the neurobiological adaptations that may underlie these effects. Finally, I will
highlight how my work will add to the contemporary literature on long-lasting effects of adolescent social experiences on adult behavior and physiology.

1.2. Defining adolescence

1.2.1. The age span of adolescence

Adolescence is a developmental period described as the gradual transition between childhood and adulthood (Spear, 2000). Human adolescence has been defined as the period between the ages of 10 – 19 years, although some researchers have considered ages up to 25 as late adolescence (Spear, 2000; WHO, 2010). While it’s difficult to characterize the precise ontogenetic time course of adolescence in animals, many of the behavioral, cognitive, and neurobiological alterations that occur during human adolescent development (discussed below) are conserved across a variety of mammalian species, ranging from rodents to non-human primates (Spear, 2000). In rodents, adolescence is typically subdivided into 3 distinct epochs, namely prepubescence/early adolescence from postnatal day (PND) 21 – 34, mid-adolescence from PND 34 – 46, and late adolescence from PND 46 – 59. Rodents are considered sexually mature young adults at PND 60 (McCormick, Mathews, Thomas, & Waters, 2010; Tirelli, Laviola, & Adriani, 2003). It is, however, worth noting that many adolescent-specific developmental transitions in brain and behavior occur from PND 28-42 and this age-range is often used as a conservative estimate of rodent adolescence (Spear, 2000). Given the brevity of rodent adolescence and the fact that individuals from a variety of species undergo similar developmental challenges as they attain skills necessary for independence, rodent models
have become increasingly popular for research into the neurobiological processes that drive adolescent behavioral changes and the factors that impinge upon development and lead to psychopathology.

1.2.2. Adolescence vs. puberty

The terms adolescence and puberty are often used interchangeably, but there are important distinctions between them. Adolescence has been defined as the developmental period between the onset of sexual maturation and attainment of adult roles and responsibilities (Dahl & Spear, 2004). In this sense, adolescence is period of gradual transitions during which physiological, behavioral, affective, and cognitive maturation culminates in attainment of the skills necessary for independence and management of adult social roles (Dahl & Spear, 2004; Spear, 2000). Puberty is often considered a marker for the onset of human adolescence (Forbes & Dahl, 2010; Sisk & Foster, 2004). Puberty refers to activation of the hypothalamic-pituitary-gonadal axis which results in heightened levels of gonadotropin (luteinizing hormone + follicle stimulating hormone) release from the pituitary gland (Sisk & Foster, 2004). The pubertal rise in gonadotropin levels stimulates ovarian estrogen secretion and initiation of ovulatory menstrual cycles in girls as well as testicular androgen secretion and spermatogenesis in boys. Pubertal development also involves secretion of adrenal androgens, rapid changes in body size and composition, and the emergence of secondary sex characteristics (Buck Louis et al., 2008). Following pubertal development an individual will be sexually and reproductively competent. However, these physiological changes are insufficient to provide the broader set of skills and knowledge necessary to take on the social roles and responsibilities of
adulthood (Dahl & Spear, 2004). Consequently, puberty is but one of many adolescent transitions into adulthood. An in-depth discussion of each component of adolescent development is beyond the scope of this dissertation, but a general consensus has emerged in the literature. Adolescent gonadal, behavioral, affective, and cognitive maturation are distinct processes mediated by social, cultural, and familial influences on developing biological systems (Dahl & Spear, 2004; Sisk & Foster, 2004). The current dissertation will focus specifically on the impact of social challenges during adolescent development on psychopathology-related behaviors.

1.2.3. Rodent models of adolescence

Animal models are useful tools for generating and testing hypotheses regarding the mechanisms that mediate adolescent development (Gottlieb, Carolina, & Lickliter, 2004). However, the ability of findings from rodent research to effectively translate into hypotheses regarding the human condition critically depends on the validity of the model. In order to develop useful hypotheses from animal models several criteria must be satisfied. First, there must be similarities in the ontology of adolescent social behavior in terms of developmental history and neurobiological/hormonal characteristics (face validity). There should also be homology between the psychosocial and physiological factors that shape rodent and human adolescent social development and the consequences of disruptions to development (construct validity). Finally, disruption to rodent social development should lead to behavioral phenotypes and neurobiological alterations similar to those observed in humans (predictive validity) (Willner, 1991). Although assessment is
ongoing, numerous similarities between human and rodent adolescent social and biological development have been reported (reviewed in Spear, 2000). As will be discussed throughout this chapter, there is ample evidence that rodent models of adolescent development possess good face, construct, and predictive validity.

In the next sections I will review the literature on human and rodent adolescent social development. I will then present findings suggesting that disruptions in adolescent social development (i.e., social stress) are associated with increased risk for affective disorders and nicotine use. Finally, parallel findings from animal models of social stress will be discussed. Taken together, the available literature suggests that rodent models of adolescent social stress are ethologically relevant and useful for developing hypotheses regarding mechanisms underlying the development of co-morbid affective disorders and nicotine use.

1.3. Adolescent social development in humans and animal models

Human adolescence is associated with a number of significant behavioral changes such as increased novelty-seeking, impulsivity, and risk-taking (Boyer, 2006; Steinberg, 2004). Alterations in the quantity and quality of social interactions and affiliation with peers are also prominent behavioral changes exhibited by adolescents. Adolescent relationships with peers acquire increased affective and motivational salience (Steinberg & Morris, 2001). As such peers can have a significant influence on socioemotional development (Rubin, Bukowski, & Parker, 2006). The majority of adolescent social interactions are peer-directed and represent a significant source of positive experiences
for teens (Csikszentmihalyi, Larson, & Prescott, 1977). Peer groups also become considerably larger and there is less adult supervision (Rubin et al., 2006). The influence of peers on adolescent development can also be negative (Mounts & Steinberg, 1995; Wentzel & Caldwell, 1997). Peer influences are a major factor contributing to adolescent engagement in risk-taking, novelty-seeking, and other maladaptive behaviors (Berndt, 1979; Steinberg, 2004; Urberg, Değirmencioğlu, & Pilgrim, 1997).

Rodents exhibit a peer-directed social re-orientation similar to that observed in humans. Time spent in social interaction is greater in adolescent rats and mice compared to adults and the frequency of social play behavior peaks during early – mid adolescence (PND 30-40) followed by a decline around puberty (Panksepp et al., 2007; Pellis & Pellis, 1998; Primus & Kellogg, 1989; Takahashi & Lore, 1983; Terranova, Laviola, & Alleva, 1993; Thor & Holloway, 1984; Wolff, 1981). Rodent social play most often refers to play fighting which is similar in both male and female rats. Play fighting is most extensively characterized in rats. During play fighting, playful attacks are directed towards the nape of the neck and a number of defensive maneuvers are used by the recipient to avoid contact (Pellis & Pellis, 1998). Counterattacks are, in turn, launched by the recipient and these role reversals are a defining characteristic of rat play fighting (Pellis & Pellis, 1991). Notably, adult rats that have been deprived of play experiences during adolescence exhibit numerous deficits in cognitive and social competency including hyper-defensiveness when approached by conspecifics and difficulty coordinating motor movements with social partners (Pellis & Pellis, 2007).
Considerable differences in juvenile play behaviors exist across species. Play fighting among adolescent inbred C57BL/6J mice occurs at rates 10-15 times lower than rats (Pellis & Pasztor, 1999; Pellis & Pellis, 1997). Notably, outbred adolescent male CD-1 mice begin to exhibit adult-like aggressive agonistic behaviors by PND 30 and rates plateau by PND 35 at which point stable social dominance-submission relationships emerge (Terranova, Laviola, de Acetis, & Alleva, 1998). This lack of qualitative differences between adolescent and adult aggression and the absence of role reversals during aggressive encounters are in marked contrast to rats. Mice do, however, exhibit some forms of social play during adolescence (Laviola & Alleva, 1995; Terranova et al., 1993; Wolff, 1981). These and other affiliative behaviors, such as allogrooming or play solicitation, peak between PND 23-32 in CD-1 and C57BL/6J mice (Laviola & Alleva, 1995; Panksepp et al., 2007; Terranova et al., 1993). Importantly, isolation housing increased levels of overt aggression as well as affiliative and soliciting behaviors in adolescent CD-1 mice relative to group housed mice (Terranova et al., 1993, 1998). Thus, social experiences are an important component of both mouse and rat socioemotional development during adolescence.

When comparing the ontogeny of human and rodent adolescent social behavior several similarities emerge. Most notably, adolescent rats and mice exhibit heightened levels of species-specific social behaviors during adolescence relative to other developmental stages. Similar to human adolescents, social interactions with unfamiliar conspecifics also appear to be highly salient in rodents. The motivational salience associated with social play is particularly interesting in rats as it appears to be stronger
for adolescents than it is in adults (Calcagnetti & Schechter, 1992; Douglas, Varlinskaya, & Spear, 2004; Humphreys & Einon, 1981).

1.4. Adolescent social stress may contribute to co-morbid affective disorders and nicotine use in humans and animal models

Human epidemiological studies have provided evidence of a prominent rise in the incidence of affective disorders including anxiety and depression during adolescence (Kessler, Berglund, & Demler, 2005). Adolescent social interactions have a considerable influence on the development of internalizing problems that characterize affective disorders which may be compounded by increased concerns regarding social acceptance and peer rejection (Rubin et al., 2006). The importance of social relationships on the development of later-life internalizing problems is exemplified by research on social withdrawal, defined as consistent display of solitary behavior in the presence of both familiar and unfamiliar peers (Coplan, Prakash, O’Neil, & Armer, 2004; Rubin & Coplan, 2004). Social withdrawal is moderately stable from childhood to adolescence. Consequently, the establishment of normal social relationships and the development of social and cognitive skills derived from peer interactions are impeded. This may account for increased rates of peer-rejection observed in these children (Rubin et al., 2006; Rubin, Hymel, & Mills, 1989). Social stressors such as bullying and victimization, which also predict internalizing problems, emerge during early adolescence and socially withdrawn children are at greater risk for victimization (Esplage, Bosworth, & Simon, 2000; Rubin et al., 2006). Socially withdrawn children exhibit increased anxiety, depression, and isolation from peers during adolescence and adulthood (Rubin, Burgess, Kennedy, &
Stewart, 2003). These findings raise the possibility that disruptions in adolescent social development (i.e., social stress) may contribute to the development of affective disorders.

A concomitant increase in the incidence of affective disorders accompanies a rise in the risk of smoking initiation and development of nicotine dependence during adolescence. This is evidenced by the fact that nearly all adult smokers start before the age of 20 (Substance Abuse and Mental Health Services Administration, 2011). Importantly, there is a strong association between affective disorders and nicotine use (Grover, Goodwin, & Zvolensky, 2012; Jamal, Willem Van der Does, Cuijpers, & Penninx, 2012; Koenen et al., 2005; Richardson, He, Curry, & Merikangas, 2012; Swendsen et al., 2010). Findings from prospective studies indicate that adolescent social experiences are important predictors of risk for nicotine use. For example, an adolescent’s initial onset of cigarette use and transition to current smoking status are influenced by the smoking status of their friends (Ennett & Bauman, 1994; Urberg et al., 1997). However, social stressors also contribute to smoking risk. For example, adolescents that had low social status in their school, which presumably indicates exposure to social stressors, were at greater risk for initiating smoking than adolescents with high social status (Finkelstein, Kubzansky, & Goodman, 2006).

One mechanism by which adolescent stress may increase risk for smoking is through activation of the HPA axis stress response (Donny et al., 2000; Pomerleau & Pomerleau, 1990). In adults, stress increases smoking intensity (Rose, Ananda, & Jarvik, 1983) and many smokers indicate that nicotine relieves stress and anxiety (Pomerleau & Pomerleau, 1991; Pomerleau, Turk, & Fertig, 1984). These classic findings suggest that
stress may increase the reinforcing properties of nicotine (Pomerleau & Pomerleau, 1990, 1991). Glucocorticoids are believed to mediate many of the pharmacological properties of nicotine, including its reinforcing properties, and contribute to the development of dependence (Pauly, Grun, & Collins, 1992; Pomerleau & Pomerleau, 1991). Although, precise causal mechanisms underlying this association remain unclear, glucocorticoid-mediated changes in the expression of nicotinic acetylcholine receptors may contribute to these findings (Pauly et al., 1992). Interestingly, both affective disorders and smoking initiation have been associated with adolescent stress and elevated stress hormone levels in prospective clinical studies (Finkelstein et al., 2006; Rao, Hammen, Ortiz, Chen, & Poland, 2008).

The preclinical literature suggests that, like humans, adolescent social stress increases anxiety- and depression-related behaviors rodents (Sachser, Durschlag, & Hirzel, 1998; Sachser, Kaiser, & Hennessy, 2013). The most commonly used social stress method in adolescent rodents is social isolation. Social isolation typically begins around weaning (~PND 21) and lasts throughout adolescence and into adulthood. Studies have shown that social isolation increases anxiety- and depression-related behavior in rats and mice (Arndt et al., 2009; Bartolomucci et al., 2004; Hong et al., 2012; Palanza, Gioiosa, & Parmigiani, 2001; Pisu et al., 2016; Ros-Simó & Valverde, 2012). However, results are highly variable as they are dependent on the animals sex and genetic background (Arndt et al., 2009; Palanza et al., 2001). Furthermore, social isolation has been criticized for its lack of validity with regard to human adolescent social experiences (McCormick & Green, 2013).
Several recent adolescent social stress protocols have been described as more ethologically–relevant models of disruption to human social development (McCormick et al., 2015a; Schmidt, Sterlemann, & Müller, 2008). For example, social instability stress, which consists of continuous and repeated exposure to novel social partners, has been a useful rodent model of adolescent social stress. In rats exposure to social instability stress from PND 30-45 increased anxiety- and depression-related behaviors and altered adult social competency in adult male and female Long Evans (Green, Barnes, & McCormick, 2013; Mathews, Wilton, Styles, & McCormick, 2008; McCormick et al., 2015, 2008). Similar results have been observed in male and female CD-1 mice following exposure to social instability stress from PND ~28-80 (Scharf et al., 2013; Schmidt et al., 2007; Schmidt, Scharf, Liebl, et al., 2010; Schmidt, Trümbach, et al., 2010; Sterlemann et al., 2008). These effects were attributable to stress-induced HPA axis dysregulation and were prevented by concurrent treatment with a corticotrophin-releasing hormone receptor type 1 antagonist (Schmidt et al., 2007). However, it is worth noting that several studies have found no effect of this social instability stress paradigm in CD-1 mice (Boleij et al., 2014; Scharf et al., 2013; Schmidt, Scharf, Sterlemann, et al., 2010). These inconsistent results have been associated with individual differences in stress-susceptibility that may arise from genetic variability among individuals in this outbred strain (Schmidt, Trümbach, et al., 2010). Another common social stress paradigm, social defeat stress, has been proposed to model bullying and social subordination experienced by human adolescents (McCormick & Green, 2013). Although results are more variable, social defeat stress
during adolescence has also been shown to increase adult anxiety- and depression-related behaviors in male rats and mice (Bourke & Neigh, 2011; Iñiguez et al., 2014; Vidal et al., 2007; Watt, Burke, Renner, & Forster, 2009).

There are few studies that have investigated the impact of adolescent social stress on subsequent nicotine responses and results have been inconsistent. In one study, there was no change in locomotor sensitization to repeated nicotine injections or voluntary oral nicotine consumption in adult rats that were deprived of social play in adolescence (Himmler et al., 2014). Alternatively, exposure to social instability stress during mid-adolescence blunted locomotor sensitization in late-adolescent (PND 58) female rats (McCormick & Ibrahim, 2007), had no effect on sensitization at a lower dose in adulthood (PND 69) ~3.5 weeks after stress exposure (McCormick, Robarts, Kopeikina, & Kelsey, 2005), and augmented sensitization in adult (PND 80) females tested ~5 weeks after stress exposure (McCormick et al., 2004). Finally, there were no effects of adolescent social defeat or even a non-social stressor (i.e. chronic restraint stress) on nicotine-induced locomotion or self-administration in male rats (Cruz, DeLucia, & Planeta, 2008; Zou, Funk, Shram, & Lê, 2014). Thus, adolescent social experiences can impact later life nicotine responses. However, these effects will be dependent on genetic background, sex, and perhaps even the amount of time that has elapsed between the conclusion of stress exposure and nicotine testing.

The literature summarized above suggests that social behavior may be a critical mediator of normative adolescent development. During early adolescence peer-directed interactions become the most prominent feature of one’s social experiences
(Csikszentmihalyi et al., 1977). Furthermore, social experiences with peers acquire heightened affective and motivational salience (Rubin et al., 2006; Steinberg & Morris, 2001). Interactions with peers are thought to promote the development of social skills as adolescents transition to independence (Rubin et al., 2006; Spear, 2000). As such, social stressors including bullying, victimization, peer rejection, and social isolation may promote maladaptive socioemotional development (McCormick, Merrick, Secen, & Helmreich, 2007; Rubin et al., 2006; Spear, 2000, 2009). Moreover, disruptions in normative social development may contribute to development of comorbid affective disorders and nicotine use. Augmented emotionality and physiological responses to social stress exhibited by adolescents may increase vulnerability to affective disorders and risk for smoking among at-risk individuals (Rao, Hammen, London, & Poland, 2009a; Spear, 2009). Taken together animal models of social stress can be useful for studying the mechanisms underlying these effects.

1.5. Adolescent maturation of the stress-susceptible neurobiological systems relevant to co-morbid affective disorders and nicotine use

In this section, I will review the literature on human and rodent adolescent development of stress-susceptible corticolimbic brain structures that regulate reward behaviors, emotion reactivity, and stress responses. Maturational changes in both structure and function will be discussed at the gross anatomical and cellular levels. I will then present findings from the literature that suggest these maturational processes are disrupted by adolescent social stress and may be important for affective disorder and nicotine use comorbidity.
1.5.1. Structural maturation of stress-susceptible corticolimbic brain regions

Wide-spread neuroanatomical and functional alterations have been described in the adolescent brain (Blakemore, 2008; Casey, Getz, & Galvan, 2008; Crone & Dahl, 2012; Nelson, Leibenluft, McClure, & Pine, 2005). Longitudinal neuroimaging studies in normally developing children and adolescents have indicated that there are complex changes in gray and white matter volume throughout adolescence. In general, cortical gray matter volume exhibits an inverted-U shaped pattern and there is a linear increase in white matter volume that continues through adulthood (Giedd, 2004; Gogtay et al., 2004; Sowell, Thompson, & Toga, 2004). These studies demonstrated that cortical regions mediating basic functions, such as sensory and motor cortices, mature early in adolescence. The prefrontal cortex (PFC) is responsible for integration of these functions but does not mature to adult levels until late-adolescence (Gogtay et al., 2004). Morphological and functional maturation of the adolescent brain likely contributes to behavioral changes observed during adolescence.

Volumetric changes subcortical limbic regions known to regulate stress-responsivity, emotional reactivity, and reward behaviors have also been reported in prospective studies of typically developing human adolescents (Giedd et al., 1996, 1999; Gogtay et al., 2006; Sowell, Thompson, Holmes, Jernigan, & Toga, 1999). For example, hippocampal and amygdala volume increase early in adolescence (Giedd et al., 1996; Goddings et al., 2014). In contrast, nucleus accumbens (NAc) volume decreases (Goddings et al., 2014; Sowell, Trauner, Gamst, & Jernigan, 2002). The PFC exerts inhibitory control over these subcortical limbic regions (Casey et al., 2008; Ulrich-Lai &
Herman, 2009). Thus, protracted maturation of top-down inhibitory control mediated by the adolescent PFC, when coupled with early maturation of subcortical limbic regions, may account for adolescent-specific changes in reward behaviors, emotion reactivity, and stress responses (Casey et al., 2008). Indeed, enhanced sensitivity to reward has been associated with exaggerated ventral striatal (the ventral striatum includes the NAc) activation among adolescents relative to other ages (Galvan et al., 2006; Geier, Terwilliger, Teslovich, Velanova, & Luna, 2010; Van Leijenhorst et al., 2010). Heightened amygdala responses to threat cues were also observed in adolescents relative to children and adults. In this study, reduced functional connectivity between the ventral PFC and amygdala was also associated increased anxiety (Hare et al., 2008).

Preclinical studies have provided valuable insight into the mechanisms that mediate human adolescent brain development. In general, trajectories of adolescent cortical development appear to be highly conserved across species. Of interest are changes in the volume and neuronal morphology of corticolimbic brain regions relevant to stress-responsivity, emotional reactivity, and reward behaviors. Like humans, rat medial PFC (mPFC) grey matter volume exhibits an inverted-U shaped trajectory and white matter volume increases across adolescent development (Koss, Belden, Hristov, & Juraska, 2014; Markham, Morris, & Juraska, 2007). At the cellular level, the initial rise in mPFC volume likely reflects a period of synapse over-production. There is a linear increase in rat mPFC synaptic density from PND 20-40 (Andersen & Teicher, 2004). Developmental changes in synaptic density are associated with an increase in dendritic length, branching, and spine density (Koss et al., 2014). Increased mPFC white matter volume is associated
with a rise in the number of glial cells in the dorsal mPFC (Markham et al., 2007). A period of cortical thinning in late-adolescence and adulthood is associated with a marked reduction in synapse density (PND 40-100) (Andersen & Teicher, 2004). Synaptic pruning may be the result of dendritic retraction and the loss of dendritic spines (Koss et al., 2014). However, programmed cell death also contributes to adolescent cortical thinning. The ventral mPFC of adult rodents exhibits a significant reduction in the number of neurons relative to adolescents (Juraska & Markham, 2004; Markham et al., 2007).

The hippocampus exhibits a profound capacity for structural plasticity throughout life and has an important role in regulation of mood, physiological responses to drugs of abuse, and HPA axis responses to stress (Bannerman et al., 2014; Britt et al., 2012; McEwen, Eiland, Hunter, & Miller, 2012; Ulrich-Lai & Herman, 2009). As with the mPFC, trajectories of hippocampal development are similar between humans and animals. For example, hippocampal volume is greater in adult rats and mice relative to early- and mid-adolescent animals (Isgor, Kabbaj, et al., 2004; Koshibu, Levitt, & Ahrens, 2004; Yildirim et al., 2008). There are several factors that contribute to the age-related structural changes reported in the rodent hippocampus. In male rats, synaptic density rises from PND 40-60 followed by a reduction from PND 60-80 (Andersen & Teicher, 2004). A similar curvilinear trajectory was found for changes in dendritic spine density on dorsal CA1 pyramidal neurons in male mice (Meyer, Ferres-Torres, & Mas, 1978). In females, dendritic branching of ventral hippocampal pyramidal neurons increases from PND 44-51 followed by a reduction in branching from PND 51-55
Although, there were no age-related differences in ventral hippocampal spine density, the number and density of dorsal hippocampal dendritic spines decreased from PND 35-49 (Chowdhury et al., 2014; Yildirim et al., 2008).

The amygdala is heavily implicated in regulation of social behaviors, anxiety-related behavior, reward processing, and the HPA axis response to stress (Janak & Tye, 2015; Ulrich-Lai & Herman, 2009). Again, the overall trajectory of volumetric growth is similar in the human and rodent amygdala during adolescence. Increased basolateral and medial amygdala volume has been reported in late-adolescent and adult rodents relative to early adolescent animals (Cooke, 2011; Romeo & Sisk, 2001; Rubinow & Juraska, 2009). Volumetric changes in the BLA (BLA) are associated with an increase in synaptic density, dendritic arborization, and spine density for both males and females during early adolescence (Cunningham, Bhattacharyya, & Benes, 2002; Koss et al., 2014; Tsai et al., 2013). Consistent with the overall volumetric increase reported in human and rodent amygdala, there were no differences in synaptic density, dendritic length, or spine density between late-adolescent and early-adult rats (Andersen & Teicher, 2004; Koss et al., 2014).

Maturational changes in the functional connectivity of corticolimbic brain regions is well-documented among human adolescents. Like humans (Hare et al., 2008), connectivity between the amygdala and mPFC also matures throughout adolescence in rodents. Neuroanatomical tracing studies have demonstrated a progressive increase in the density of BLA terminals in the mPFC. The process of mPFC innervation starts prior to adolescence and continues into adulthood (Cunningham et al., 2002). BLA projection
neurons are glutamatergic and frequently synapse on GABAergic interneurons in mPFC (Cunningham et al., 2002). Thus, stimulation of BLA efferents likely causes the interneurons to inhibit mPFC pyramidal neurons. This process may play a role in the development of top-down inhibitory control exerted over the amygdala by the PFC (Casey et al., 2008). Interestingly, innervation of the BLA by prelimbic and infralimbic mPFC projection neurons peaks by early-adolescence followed by considerable pruning from late-adolescence to adulthood (Cressman et al., 2010). Developmental shifts in BLA-mPFC connectivity support data from human imaging studies demonstrating early development of subcortical limbic structures relative to delayed maturation of top-down control systems including the PFC (Casey et al., 2008).

The NAc is well-known for its role in reward processing following stimulation by drugs of abuse as well as natural reinforcers (Scofield et al., 2016). Recent studies suggest that it also plays a prominent role in regulating emotional behavior (Nestler & Carlezon, 2006). Importantly, excitatory projections from the mPFC, amygdala, and hippocampus converge onto individual NAc medium spiny neurons (MSNs) which are also under modulatory control by overlapping dopamine inputs (Britt et al., 2012; Grace, Floresco, Goto, & Lodge, 2007; Scofield et al., 2016). As such, the NAc is poised to integrate information from stress-susceptible subcortical brain regions and shape relevant behavioral responses. It is, therefore, not surprising that stress-induced alterations in function of the NAc MSNs have been implicated in drug addiction and affective disorders (Andersen & Teicher, 2008; Nestler & Carlezon, 2006; Scofield et al., 2016). The structural mechanisms associated with volumetric changes in the NAc during human
adolescent development (Goddings et al., 2014; Sowell et al., 2002) are less obvious than those of the previously discussed corticolimbic regions. In one study, the density of newly proliferated cells in the NAc was greater in adolescents relative to adults (Staffend, Mohr, Doncarlos, & Sisk, 2014). However, the majority of synapses in the NAc are already present by PND 21 (Hattori & McGeer, 1973; Sharpe & Tepper, 1998). There is some synaptogenesis associated with dendritic arborization and dendritic spine formation that continues until ~PND 30 (A. K. Butler, Uryu, Rougon, & Chesselet, 1999; Sharpe & Tepper, 1998; Tepper, Sharpe, Koos, & Trent, 1998). On the other hand, alterations in excitatory synaptic transmission onto MSNs are prominent during mid-adolescence. Specifically, membrane resistance and neuronal excitability decreases to adult levels (Kasanetz & Manzoni, 2009). These changes are associated with shifts in surface expression of AMPA and NMDA receptor subunits (Butler et al., 1999; Tepper et al., 1998). Finally, developmental changes in connectivity were found as the density of mPFC projections terminating in the NAc increases throughout adolescent development (Brenhouse, Sonntag, & Andersen, 2008).

1.5.2. **Maturation of neurochemical systems in stress-susceptible corticolimbic brain regions**

Structural and functional changes in the adolescent brain are accompanied by maturation of several neurochemical systems. When considering the neurobiological mechanisms that mediate developmental changes in reward behaviors, emotion reactivity, and stress responses, robust maturation of the dopamine (DA) and nicotinic acetylcholine receptor (nAChR) systems is particularly interesting. Maturational changes in brain
regions and neurotransmitter systems, other than those discussed below, may contribute to the development of adolescent-typical behavior, but are beyond the scope of this review. I will review the literature on maturation of the DA and nAChR systems because of their potential relevance to the development of comorbid nicotine use and affective disorders following exposure to adolescent stress.

During early and mid-adolescence, a rise in dopaminergic innervation of the NAc has been inferred from observations of increasing tyrosine hydroxylase and DA transporter immunoreactivity (Naneix, Marchand, Di Scala, Pape, & Coutureau, 2012; Tarazi, Tomasini, & Baldessarini, 1998). DA innervation of the NAc is associated with a concomitant rise in extracellular DA levels (Andersen & Gazzara, 1993). Dopaminergic innervation of the mPFC is delayed, relative to the NAc, and reaches adult levels in early adulthood (Cao, Lotfipour, Loughlin, & Leslie, 2007; Kalsbeek, Voorn, Buijs, Pool, & Uylings, 1988; Naneix et al., 2012). Extracellular DA levels, DA synthesis, and DA turnover in the mPFC peak by mid-adolescence, but decline by adulthood (Teicher et al., 1993). In general, these data fit with human studies demonstrating delayed maturation of the mPFC relative to subcortical limbic brain regions.

An inverted-U shaped pattern of D1 and D2 DA receptor (D1R and D2R, respectively) binding site density has also been reported in the NAc and mPFC (Andersen, Thompson, Rutstein, Hostetter, & Teicher, 2000; Naneix et al., 2012). DA receptor density in the NAc peaks in early-adolescence, but peak mPFC receptor density does not occur until mid to late-adolescence (Andersen et al., 2000; Naneix et al., 2012). Receptor density declines to adult levels in both regions following their respective peaks.
(Andersen et al., 2000; Naneix et al., 2012). In the mPFC, D1R over-production and pruning occurs primarily on glutamatergic projection neurons that terminate in the NAc with no change in expression on GABAergic interneurons (Brenhouse et al., 2008). Therefore, the adolescent mPFC may exhibit greater D1R-mediated excitability without changes to inhibitory transmission. In the mPFC, D2R signaling inhibits GABAergic interneuron activity, but this response does not emerge until late-adolescence (O’Donnell, 2010; Tseng & O’Donnell, 2007). Maturation of the adolescent DA system is essential for proper development (Andersen, 2003; Spear, 2000). Importantly, cortical DA signaling regulates the attribution of contextual salience to novel stimuli and enhances stress-responsivity (Brenhouse et al., 2008; Lyss, Andersen, Leblanc, & Teicher, 1999). As such, stress-induced disruption this processes is believed to contribute to the emergence of affective disorders and increase susceptibility to nicotine addiction (Andersen & Teicher, 2008; Burke & Miczek, 2014; Yuan, Cross, Loughlin, & Leslie, 2015).

The nAChR system also matures during adolescence. Maturation of nAChRs is especially interesting in light of their functional interactions with the adolescent DA system. Neuronal nAChRs are pentameric ligand-gated ion channels that are classified into two broad categories. Homomeric nAChRs are composed of the same subunit (i.e., α7) and heteromeric receptors possess a combination of α and β subunits (Dani & Bertrand, 2006; McGehee, 1999). A wide variety of nAChR subtypes arise from different subunit combinations which results in a diverse array of functional and pharmacological properties (Dani & Bertrand, 2006; McGehee, 1999).
Neuronal nAChRs are expressed in neurobiological circuits implicated in nicotine dependence including the mesolimbic DA system (Gotti & Clementi, 2004; Placzek, Zhang, & Dani, 2009). Nicotine-induced activation of nAChR in the ventral midbrain stimulates DA release in a variety of targets throughout the developing adolescent brain including the NAc, hippocampus, amygdala, and mPFC (Feduccia, Simms, Mill, Yi, & Bartlett, 2014; Gotti, Zoli, & Clementi, 2006; Grady et al., 2007; Placzek et al., 2009). The most common nAChR subtypes expressed in the central nervous system are those containing α4 and β2 subunits (α4β2*; * indicates the presence of additional subunits) (Dani & Bertrand, 2006). The α4β2* nAChRs exhibit high affinity for nicotine (Dani & Bertrand, 2006). They mediate many of nicotine’s reinforcing properties and they influence anxiety- and depression-related behavior (Picciotto & Kenny, 2013). The α7 nAChRs are another subtype that is widely expressed throughout the brain. These receptors have a lower affinity for nicotine and rapidly desensitize upon exposure to high concentrations of nicotine (Dani & Bertrand, 2006). The α7 receptors have also been shown to modulate nicotine reinforcement, albeit more subtly than α4βb2* nAChRs (Picciotto & Kenny, 2013). It should be mentioned that several other subunits are implicated in nicotine-induced DA release (i.e., β3, α6) and anxiety- and depression-related behavior (i.e., β4, α5) (Picciotto & Kenny, 2013).

The expression and function of the various nAChR subtypes are tightly regulated during postnatal development and possess distinct spatial patterns throughout the central nervous system (Gotti et al., 2006). Developmental changes in nAChR subtype expression have been reported in the adolescent amygdala, mPFC, hippocampus, and
NAc. For example, α4β2* nAChR density is higher in the NAc, hippocampus, and mPFC of mid-adolescent rats relative to adults. Increased α7 nAChRs density was also reported in the adolescent amygdala, ventral hippocampus, and mPFC relative to adults, but no difference was found in the NAc (Doura, Gold, Keller, & Perry, 2008). Finally, the expression of α5 and α6 mRNAs are higher in the early-adolescent rat ventral tegmental area (VTA) relative to adults (Azam, Chen, & Leslie, 2007).

The effects of nicotine on neuronal activity have provided more insight into maturation of the adolescent nAChR system. For example, there are age-related differences in α4β2* nAChR functionality measured in vitro. Adolescent rats and mice exhibit greater nicotine-induced $^{86}$Rb$^+$ efflux from striatal, mPFC, and hippocampal synaptosomes as compared to adults (Britton, Vann, & Robinson, 2006; Kota, Martin, Robinson, & Damaj, 2007). Interestingly, α4β2* nAChR functionality exhibits an inverted-U shaped developmental trajectory which peaks during mid-adolescence and declines to adult levels thereafter (Britton et al., 2006). In regard to nAChR modulation of DA systems, the effects of nicotine on DA release are more potent in the adolescent ventral striatum compared to other ages (Azam et al., 2007). Finally, VTA DA neurons are more sensitive to nicotine induced long-term potentiation (LTP) in adolescent mice relative to adults (Placzek et al., 2009). This ability of addictive drugs to stimulate LTP in VTA DA neurons correlates with a number of addiction-related behaviors (Saal, Dong, Bonci, & Malenka, 2003).

Taken together the literature summarized above indicates that the adolescent brain is subject to widespread maturational changes which are highly conserved across species.
Rodent studies have demonstrated concomitant maturational changes in the adolescent DA and nAChR systems. Furthermore, there is considerable cross-talk between these systems. Not surprisingly, both are implicated in regulation of nicotine responses and affect. As such, social stress-induced disruption to the development of these systems and their function in stress-susceptible corticolimbic brain regions may contribute to the development of comorbid nicotine use and affective disorders.

1.6. Development of adolescent stress reactivity

Accumulating research suggests that developmental changes the physiological stress response may contribute to the unique vulnerability of the adolescent brain to stress. Puberty-related changes in peripheral biomarkers of the neurobiological systems that mediate emotion and stress reactivity have recently been characterized in normally developing teens. For example, mid-/late pubertal adolescents exhibited greater pupillary reactivity during an emotional word identification task, better memory for emotional words than neutral words, and reported higher negative affect compared to pre-/early pubertal adolescents (Silk et al., 2009). Mid-/late pubertal adolescents also exhibited potentiation of the auditory motor reflex during presentation of pleasurable stimuli which was not present in pre-/early pubertal adolescents (Quevedo, Benning, Gunnar, & Dahl, 2009). These findings are in agreement with the results of human neuroimaging studies and suggest that adolescent’s exhibit heightened reactivity in corticolimbic brain regions that modulate the autonomic nervous system and reward processing.
Adolescence-associated increases in physiological indicators of stress reactivity accompany changes in emotional reactivity. Basal cortisol levels were significantly higher in 15-year-olds compared to 9 to 13-year-old children and adolescents (Gunnar, Wewerka, Frenn, Long, & Griggs, 2009). These results were consistent with several studies indicating that adolescent development is associated with an elevated homeostatic set-point for basal HPA axis activity (Adam, 2006; Legro, Lin, Demers, & Lloyd, 2003; Schiefelbein & Susman, 2006; Stroud et al., 2009). With respect to social stressors, cortisol production and autonomic reactivity in response to the Trier Social Stress Test, a commonly used laboratory stressor task characterized as an unpredictable and uncontrollable social threat (Dickerson & Kemeny, 2004), were significantly greater in older adolescents (13+) compared to younger adolescents and children (9 – 12 years old) (Gunnar et al., 2009; Stroud et al., 2009). Increased salivary cortisol was also observed among older adolescents following a social performance stressor and while experiencing negative emotions such as anger, worry, and stress (Adam, 2006; Klimes-Dougan et al., 2001). Interestingly, adolescent changes in stressor reactivity appear to be stimulus specific. Whereas no ontogenetic differences in cortisol reactivity were observed following peer-rejection, adolescents exhibited significantly greater cardiovascular (systolic blood pressure) and autonomic (salivary alpha amylase) responses than younger children (Stroud et al., 2009). These adolescent-specific changes may be particularly detrimental for “at-risk” individuals such as those exhibiting high levels of anxiety or anxious temperament (Gunnar et al., 2009; Klimes-Dougan et al., 2001; Quevedo et al., 2009). As such, several researchers have postulated that normative developmental alterations in corticolimbic brain regions may open up periods of vulnerability to the
The rodent HPA axis matures during adolescence resulting in heightened stress-reactivity similar to that observed in humans. Adolescent rodents exhibit functionally immature HPA axis negative feedback regulation when exposed to a variety of physical and psychological stressors (McCormick & Mathews, 2010; Romeo, 2010). For example, when exposed to acute stress early to mid-adolescent animals (< PND 28-50) will exhibit protracted adrenocorticotropic hormone (ACTH) and corticosterone (CORT) production compared to adults (PND 70) (Foilb, Lui, & Romeo, 2011; Goldman, Winget, Hollingshead, & Levine, 1973; Lui et al., 2012; Vázquez & Akil, 1993). Unlike human adolescents, baseline ACTH and CORT levels in adolescent rodents do not differ from adults (Romeo, 2010). A prior history of stress exposure also has divergent effects on HPA axis responses to stress in adolescent and adult animals. When adults are repeatedly exposed to a stressor, they will exhibit habituation as indicated by a reduction in stress-induced ACTH and CORT production (Grissom & Bhatnagar, 2009). Alternatively, early-adolescent rodents fail to habituate to repeated stress (Romeo, 2010).

Preclinical rodent studies have yet to pinpoint the precise mechanisms underlying this developmental shift in stress reactivity. Importantly, corticolimbic brain regions including the mPFC, amygdala, and hippocampus are responsible for modulating HPA axis responses to stress (Ulrich-Lai & Herman, 2009). Initially, the glucocorticoid receptor (GR) agonist, dexamethasone, was found to inhibit adult stress-induced CORT production more effectively compared to early-adolescents (Goldman et al., 1973).
Dexamethasone-induced suppression of the HPA axis is mediated by GR (Holsboer, 2000). Thus, results suggested that GR expression may be lower in the adolescent brain compared to adults. However, in both humans and rodents, corticosteroid receptor expression (both mRNA and protein levels) in HPA axis negative feedback sites including the pituitary, mPFC, hippocampus, and hypothalamus reach adult levels soon after birth and remain relatively stable thereafter (Dziedzic, Ho, Adabi, Foilb, & Romeo, 2014; Perlman, Webster, Herman, Kleinman, & Weickert, 2007; Pryce, 2008; Romeo et al., 2007; Romeo, Kaplowitz, Ho, & Franco, 2013). Peripheral mechanisms contribute to heightened stress responsivity. A recent study found that early adolescent rats produce significantly more CORT in response to ACTH injection than adults (Romeo et al., 2014). Alternative central mechanisms could include developmental changes in regulation of genomic corticosteroid receptor signaling (via downstream regulation of gene expression) or rapid non-genomic signaling (via g-protein coupled receptors in neuronal membranes). These potential mechanisms have yet to be evaluated.

There are, to my knowledge, no studies that have directly compared acute HPA axis stress responses in human adolescents and adults. However, the available literature suggests that both humans and animals experience heightened hormonal stress responses during adolescence. These findings indicate that actively maturing corticolimbic regions of the adolescent brain are exposed to significantly greater quantities of stress-hormones compared to other ages. Stress hormones, including glucocorticoids, play an important role in programming adolescent brain development (Pryce, 2008). Thus, several researchers have proposed that adolescent stress may alter the developmental trajectories
of the mPFC, hippocampus, amygdala, and NAc which could give rise to affective disorders and increase susceptibility to substance abuse (Andersen & Teicher, 2008; Romeo, 2016).

1.7. Effects of adolescent stress on the development of corticolimbic brain regions implicated in affective disorders and nicotine dependence

As previously discussed, adolescent social stress has been associated with the development of affective disorders and nicotine use in humans and rodent models. Importantly, these stress effects have been attributed to elevated glucocorticoids in prospective clinical studies. In this section, I will present the evidence that adolescent stress programs the development of corticolimbic brain regions associated with reward behaviors, emotion reactivity, and stress responses. Stress effects on development are attributable, at least in part, to elevated glucocorticoids.

1.7.1. Adolescent stress effects on the hippocampus

The effects of stress on hippocampal structure and function have received much attention following the seminal work of McEwen and colleagues. These studies were the first to demonstrate stress-induced structural plasticity in the adult mammalian brain. They found that chronic restraint stress or prolonged exposure to elevated CORT levels suppressed neurogenesis in the dentate gyrus, reduced dendritic branching, and caused a loss of dendritic spines in CA3 and dentate gyrus neurons (McEwen, Nasca, & Gray, 2016). Exposure to chronic stress during adolescence has similar effects on hippocampal structure and function. Atrophy was reported in apical dendrites of CA3 pyramidal neurons of late-adolescent rats exposed to chronic restraint stress (Eiland, Ramroop, Hill,
Manley, & McEwen, 2012). In adults, these stress-induced structural changes are reversible following a recovery period (McEwen et al., 2016). However, the effects of adolescent stress may persist well into adulthood. For example, chronic variable stress abrogated volumetric growth of all hippocampal subfields when measured after a 3 week recovery period (i.e. during adulthood) (Isgor, Kabbaj, et al., 2004). Surprisingly, adolescent social instability stress increased the survival of immature neurons in the dentate gyrus of adult male rats (McCormick et al., 2012). As for hippocampal function, induction of LTP was impaired in the hippocampus of male mice exposed to adolescent social instability stress when measured 1 year, but not 6 months, following the conclusion of stress (Sterlemann et al., 2010). In each of these studies, stress-induced changes to hippocampal structure and function were associated with impairments in hippocampus-dependent memory, anxiety, and depression-related behavior (Eiland et al., 2012; Isgor, Slomianka, & Watson, 2004; Sterlemann et al., 2010).

It is important to mention that almost all adolescent stress effects on hippocampal structure and function were reported in the dorsal hippocampus. Stress-induced alterations to ventral hippocampal (vHIP) maturation may be of greater interest when considering mechanisms for co-morbid anxiety/depression-related behavior and nicotine use. The vHIP sends glutamatergic projections to the mPFC, NAc, amygdala, and hypothalamus (Fanselow & Dong, 2010). These structures are implicated in stress-induced neuroendocrine activity, anxiety and depression-related behavior, and drug-seeking behavior (Bannerman et al., 2014; Scofield et al., 2016; Ulrich-Lai & Herman, 2009). Furthermore, stress and corticosteroids have opposite effects on the strength of
synaptic connectivity, as measured by LTP, in dorsal and ventral hippocampus (Segal, Richter-Levin, & Maggio, 2010). For example, exposure to adolescent stress impaired dorsal hippocampus LTP, but facilitated vHIP LTP (Grigoryan, Ardi, Albrecht, Richter-Levin, & Segal, 2015). Mice selectively bred for high anxiety-related behavior also exhibit enhanced excitatory synaptic drive onto vHIP CA1 pyramidal cells (Dine et al., 2015). Finally, increased extracellular norepinephrine was reported in the ventral dentate gyrus, where it promotes LTP, of rats exposed to adolescent social defeat (Watt et al., 2009). In this study, stress-induced anxiety-like behavior was correlated with vHIP norepinephrine levels (Watt et al., 2009). The role of vHIP in nicotine-related behaviors has received less attention. However, stress-induced enhancements of vHIP glutamate transmission indirectly drives VTA DA neuron population activity to increase DA release in the NAc (Valenti, Lodge, & Grace, 2011). This stress effect has been proposed to underlie vHIP-dependent enhancement of nicotine self-administration following exposure to stress (Yu & Sharp, 2015). As such, adolescent stress-induced enhancement of vHIP activity could contribute to the development of co-morbid nicotine use and affective disorders.

1.7.2. Adolescent stress effects on the prefrontal cortex

The prefrontal cortex is remarkably sensitive to stress-induced structural changes (Arnsten, 2009; McEwen et al., 2016). In adults, 3 weeks of chronic restraint stress or daily CORT injections resulted in reduced dendritic branching and spine density in the apical dendrites of layer II/III pyramidal neurons (Cook & Wellman, 2004; Liston et al., 2006; Radley et al., 2006). Stress-induced structural remodeling was associated with
impairments in mPFC-mediated cognitive functions (Liston et al., 2006); a risk factor for smoking, anxiety, and depression (Arnsten, 2009). Similar results have been found in rodents exposed to adolescent stress. Specifically, mPFC pyramidal neurons showed dendritic atrophy following chronic restraint or social isolation in early adolescence (Eiland et al., 2012; Wang, Ho, Ko, Liao, & Lee, 2012). Early adolescent social isolation also reduced mPFC synaptic density (Leussis & Andersen, 2008). Unlike adults, this effect persisted for at least 3 weeks after stress concluded (Leussis, Lawson, Stone, & Andersen, 2008). Long-lasting impairments in mPFC-mediated cognitive function were also found in adult rats exposed early adolescent stress (Tzanoulinou et al., 2015).

Adolescent social stress has a significant impact on the development of the mPFC DA. Exposure to adolescent social defeat reduced basal levels of extracellular dopamine, via D2R activation, and blunted psychostimulant-induced dopamine release in the mPFC (Burke, Forster, Novick, Roberts, & Watt, 2013; Burke, Renner, Forster, & Watt, 2010; Watt et al., 2009). Adolescent social isolation was also found to cause reductions in mPFC DA. Mice that experienced social isolation had decreased basal extracellular DA content, but only if they had increased genetic risk of psychiatric disorders (i.e., carried a transgene that disrupted DISC1 function) (Niwa et al., 2013). This effect was caused by a reduction in tyrosine hydroxylase, associated with increased methylation of the tyrosine hydroxylase gene’s promotor region, in VTA DA neurons that project to the mPFC. Stress effects were attributable to GR function because the methylation status, DA levels, and behavioral deficits were normalized by the administration of the GR antagonist RU38486 (Niwa et al., 2013). Moreover, adolescent social isolation reduced mPFC
sensitivity to the inhibitory effects of DA (Baarendse, Counotte, O’donnell, & Vanderschuren, 2013; Tseng & O’Donnell, 2007).

In the mPFC, DA receptor signaling inhibits glutamatergic projection neurons (Del Arco & Mora, 2008). Inhibition of mPFC glutamatergic projections will, in turn, reduce NAc DA responses to salient or appetitive stimuli (Grace et al., 2007). Alternatively, decreased mPFC DA signaling is coupled with excessive NAc DA activity in response to stress and psychostimulant drugs (Del Arco & Mora, 2008). Finally, excessive NAc DA activity is postulated to drive drug-seeking behavior and has been implicated in stress-induced depression-like behavior (Bagot et al., 2015; Del Arco & Mora, 2008). As such, adolescent stress-induced reductions in mPFC DA activity are a strong candidate for mechanisms that contribute to the development of comorbid affective disorders and nicotine use.

1.7.3. Adolescent stress effects on the amygdala

The effects of stress exposure during adolescence and adulthood are opposite of those found in the mPFC and hippocampus. Specifically, chronic restraint stress increased dendritic branching and the density of dendritic spines in the basolateral amygdala (BLA) of adult rats (Vyas, Mitra, Shankaranarayana Rao, & Chattarji, 2002). Similar effects were observed in adolescent rats, except that dendritic spine density was reduced following chronic restraint (Eiland et al., 2012; Padival, Blume, Vantrease, & Rosenkranz, 2015). The effects of social stress on dendritic morphology are more variable. In one study, adolescent social isolation increased dendritic branching with no
effect on dendritic length or spine density (Wang et al., 2012). Another study found a reduction in spinophilin immunoreactivity, interpreted as reduced spine density, in the basolateral and central amygdala of social isolated adolescent rats (Leussis & Andersen, 2008). Finally, chronic adolescent social instability stress decreased dendritic branching and spine density when neuronal morphology was measured in adults (Tsai et al., 2014).

These findings indicate that stress-effects on amygdala neuronal morphology may be dependent on the type of stressor used in the study. In several of these studies, increased anxiety and depression-related behavior was also observed (Leussis & Andersen, 2008; Padival et al., 2015; Wang et al., 2012).

Stress-induced alterations in development of the DA system have also been implicated in the amygdala. In particular, adolescent social isolation reduced extracellular dopamine content in the amygdala of adult rats (Heidbreder et al., 2000; Wang et al., 2012). In adults, acute stressors potently stimulate DA release in the amygdala (Inglis & Moghaddam, 1999). Dopamine signaling, via D1R activation, suppresses mPFC-mediated inhibitory control over amygdala activity (Marowsky, Yanagawa, Obata, & Vogt, 2005). As a result, amygdala disinhibition allows for the expression of anxiety-related behaviors (Pérez de la Mora, Gallegos-Cari, Arizmendi-García, Marcellino, & Fuxe, 2010). While these findings suggest that a social stress-induced reduction in amygdala DA levels would have an anxiolytic effect it is important to note that administration of a D2R antagonists (raclopride and sulpiride) to the central amygdala have anxiogenic effects in the shock-probe burying test and light-dark test (Pérez de la Mora et al., 2010). Dopamine D1R knockout mice exhibit increased anxiety-like behavior
in the elevated plus-maze as well (Short, Ledent, Drago, & Lawrence, 2006). In addition to its influence of anxiety-related behavior, the amygdala regulates natural reinforcement and drug-seeking behavior (Scofield et al., 2016). Interestingly, stress-induced enhancement of nicotine self-administration is dependent on functional connections between the BLA and NAc core (Yu & Sharp, 2015).

The effects of chronic stress on amygdala-dependent regulation of VTA DA neurons are also directly implicated in affect-related behaviors. Specifically, the BLA is hyperactive following withdrawal from chronic stress which causes inhibition of VTA DA neuron population activity (Chang & Grace, 2014). The resulting hypodopaminergic state was associated with a failure to respond to rewarding stimuli and is reminiscent of the negative affective states observed in depression and anxiety (Belujon & Grace, 2015). The effects of stress on drug-related behaviors are also associated with amygdala-dependent regulation of DA. For example, similar reductions in DA system activity and heightened negative affect are observed following withdrawal from chronic exposure to psychostimulant drugs (Belujon & Grace, 2015; Nestler & Carlezon, 2006). These findings suggest that adolescent social stress-induced amygdala hyperexcitability may contribute to the development of co-morbid affective disorders and nicotine use.

1.7.4. Adolescent stress effects on the nucleus accumbens

The NAc has been implicated in affective disorders and drug addiction due to its critical role in reward processing, motivation, and regulation of mood (Andersen & Teicher, 2008; Nestler & Carlezon, 2006; Scofield et al., 2016; Shirayama & Chaki,
However, there are only two studies, to my knowledge, which have investigated adolescent stress effects on NAc structure. In these studies, social isolation during adolescence reduced total dendritic length of NAc MSNs without influencing dendritic spine density (Alquicer, Morales-Medina, Quirion, & Flores, 2008; Wang et al., 2012). The NAc MSNs integrate afferent signals from the BLA, vHIP, and mPFC to regulate the mesocorticolimbic DA system (Britt et al., 2012; Grace et al., 2007). Together these findings suggest that adolescent social stress may impair the ability of NAc MSNs to integrate signals corticolimbic inputs and promote relevant behavioral responses (Grace et al., 2007). However, this hypothesis has not been tested.

The effects of adolescent stress on NAc DA have been studied more thoroughly. In general, NAc DA does not seem to be influenced by adolescent social stress. For example, adolescent social defeat stress had no effect on extracellular DA content in the striatum and similar results were obtained when measurements were restricted to the NAc (Vidal et al., 2007; Watt et al., 2009). No changes were found in D1R, D2R, D3R, dopamine transporter, and tyrosine hydroxylase mRNA expression in the NAc following chronic exposure to chronic social stressors (Kabbaj & Isgor, 2007). These findings suggest that adolescent stress may not increase risk for affective disorders and nicotine use by altering NAc DA system, per se.

An alternative explanation is that adolescent social stress disrupts the balance of corticolimbic inputs into the NAc (Belujon & Grace, 2015). In particular, chronic social defeat stress can induce depression-like behavior in adult rodents by upregulating VTA DA neuron burst firing (Bagot et al., 2015; Chaudhury et al., 2013; Krishnan et al., 2007).
Burst firing of VTA DA neurons shifts the balance of cortical and limbic regulation of the NAc MSNs by facilitating synaptic inputs from vHIP (via D1R activity) and suppressing mPFC inputs (via D2R activity) (Goto & Grace, 2005). Along those lines, mPFC and BLA glutamatergic inputs into the NAc are suppressed by chronic social defeat stress. Increasing synaptic transmission in either of these pathways promotes social stress resilience (Bagot et al., 2015). Although the precise mechanisms have not been investigated in adolescents, behavioral phenotypes are similar between adolescent and adult mice exposed to chronic social defeat stress (Iñiguez et al., 2014). This interpretation would be consistent with the previously reported effects of adolescent social stress on the mPFC and amygdala.

Finally, stress-induced changes in VTA DA neurons and the NAc could also directly influence behavioral and physiological responses to drugs of abuse, including nicotine, and affective behavior. In particular, the corticotropin releasing hormone (CRH) system has been implicated. Stress increases CRH release in the VTA which excites DA neurons via its type 1 receptor (CRHR1) and stimulates NAc DA release (Lemos et al., 2012; Wanat, Hopf, Stuber, Phillips, & Bonci, 2008). Recent studies have demonstrated that CRH/CRHR1 regulation of DA is critical for stress-effects on anxiety-related behavior and nicotine responses. For example, mice carrying a CRHR1 deletion in midbrain DA neurons exhibit increased anxiety-like behavior (Refojo et al., 2011). Exposure to acute stress also enhanced the rewarding properties of nicotine in adolescent rats as measured by conditioned place preference (Brielmaier, McDonald, & Smith, 2012). This stress effect was blocked by systemic administration of a CRHR1 antagonist.
(CP-154,526) (Brielmaier et al., 2012). Additional studies have found that direct application of CRHR1 antagonists into the VTA prevent cross-sensitization of cocaine-stimulated DA release and escalation of cocaine self-administration following exposure to social defeat stress (Boyson, Miguel, Quadros, DeBold, & Miczek, 2011).

1.7.5. Adolescent stress effects on the nicotinic acetylcholine receptor system

There is evidence that nAChR signaling in stress-susceptible corticolimbic brain regions could mediate the development of co-morbid nicotine use and affective disorders. However, few studies have assessed stress-induced changes in the adult nAChR system and there are none for adolescent stress. The effects of stress on the nAChR system will be reviewed here briefly, but these findings may or may not generalize to adolescents.

As discussed above (Section 1.4), nicotine-induced CORT production is proposed to mediate stress effects on smoking behavior (Pauly et al., 1992; Pomerleau & Pomerleau, 1991). However, the relationship between CORT and the effects of nicotine are bi-directional (Caggiula et al., 1998). While nicotine does stimulate CORT production (Donny et al., 2000; Lutfy et al., 2012; Matta, Fu, Valentine, & Sharp, 1998; Pomerleau & Pomerleau, 1990), CORT can also modulate nicotine responses (Caggiula et al., 1998; Pauly et al., 1992; Pauly, Grün, & Collins, 1990; Pauly, Ullman, & Collins, 1988; Robinson, Grun, Pauly, & Collins, 1996). To this end, CORT has been shown to decrease nicotine sensitivity and contribute to the development of nicotine tolerance. For example, adrenalectomy enhances nicotine-induced suppression of locomotion and hypothermia which is blocked by exogenous CORT supplementation (Pauly et al., 1990, 1988).
Conversely, mice exposed to elevated CORT levels exhibited blunted locomotor and hypothermic responses to nicotine (Pauly et al., 1990, 1988). These findings suggest that stress attenuates nicotine’s pharmacological effects and that smoking intensity is increased to overcome this effect (Caggiula et al., 1998).

Stress-induced tolerance to the pharmacological effects of nicotine may occur when elevated CORT levels alter nAChR expression. In adult mice, adrenalectomy increases expression of the α7 subunit in many brain regions including the striatum, hippocampus, midbrain, and hindbrain. Chronically elevated CORT decreased α7 expression in the hindbrain, hippocampus, and striatum (Pauly et al., 1990; Robinson et al., 1996). CORT manipulation does not influence α4β2* nAChRs (Pauly et al., 1990; Robinson et al., 1996). These studies indicate that glucocorticoids, and perhaps chronic stress, are able to influence the expression nAChRs in stress-susceptible corticolimbic brain regions such as the striatum and hippocampus. However, caution must be made when attempting to generalize these results to chronic stress exposure. The only study, to my knowledge, that has investigated the effects of chronic stress nAChR expression found that chronic restraint stress increased α7 mRNA and reduced α7 protein levels in the hippocampus (Hunter, Bloss, McCarthy, & McEwen, 2010). These effects may be related to elevated CORT levels but results were only partially recapitulated in adrenalectomized rats that were treated with the GR agonist RU28,362 (Hunter et al., 2010). Future studies will be necessary to ascertain whether similar effects are obtained across other corticolimbic brain regions following adolescent stress.
Another line of research suggests that increased cholinergic signaling contributes to affective disorders (Picciotto, Lewis, Van Schalkwyk, & Mineur, 2015). In humans, major depression is associated with increased ACh levels in many brain regions (Hannestad et al., 2013; Saricicek et al., 2012). In rodents, pharmacological inhibition of nAChR signaling, via antagonists and partial agonists, has antidepressant and anxiolytic effects (Picciotto et al., 2015). Moreover, β2* and β4* nAChRs are necessary for antidepressant effects of amitriptyline and bupropion, respectively (Picciotto et al., 2015). The antidepressant fluoxetine also reduces ACh levels by increasing acetylcholinesterase activity (Mineur et al., 2013). Conversely, mice with a point mutation that increases α4β2* nAChR function exhibit increased anxiety-related behavior (Labarca et al., 2001). Finally, upregulation of α4/α6 nAChR signaling has been shown to mediate anxiety induced by nicotine withdrawal (Molas, DeGroot, Zhao-Shea, & Tapper, 2016).

The brain areas that mediate the anxiolytic and antidepressant effects of nAChR signaling are not well characterized. However, nAChRs modulate synaptic plasticity in stress-susceptible corticolimbic brain regions (Mansvelder, Mertz, & Role, 2009). In particular, the antidepressant and anxiolytic effects of cytisine, an α4β2* partial agonist, and mecamylamine, a non-selective nAChR antagonist, may be due to α4β2* nAChR blockade in the BLA (Mineur, Somenzi, & Picciotto, 2007). These findings were extended by studies demonstrating reduced anxiety and depression-related behavior in mice with genetic knockdown of α7 and β2* nAChR signaling in the BLA (Mineur et al., 2016).
The hippocampus may be another important locus for the effects of nAChR blockade. Reduction of cholinergic tone in the hippocampus, via pharmacological and genetic inhibition of acetylcholinesterase activity, increased anxiety and depression-related behavior. These effects were reversed by fluoxetine treatment (Mineur et al., 2013). Finally, nAChR signaling in both the hippocampus and amygdala mediate susceptibility to social stress. Knockdown of the β2 nAChR subunit in the BLA prevented the effect of chronic social defeat stress on depression-like behavior (Mineur et al., 2016). In the hippocampus, acetylcholinesterase knockdown enhanced depression-like behavior observed following social defeat stress (Mineur et al., 2013).

The literature discussed above suggests that stress-induced disruption in the ACh system and nAChR signaling are sufficient to induce anxiety and depression-related behavior in adult mice and rats. Because the adolescent nAChR system undergoes profound developmental changes during adolescence, it is conceivable that chronic stress exposure could impact this process. Future studies will be necessary to determine whether nAChRs mediate the effects of adolescent stress on anxiety and depression-related behavior and determine mechanisms that may underlie these effects.

1.8. Purpose of dissertation

The literature summarized in this chapter indicates that adolescence is a period of profound developmental changes. There are many similarities in these maturational events across species. These similarities suggest that rodent models are useful tools to investigate the neurobiological mechanisms mediating adolescent development and the
effects of perturbations in these processes. In particular, rodent models of social stress provide an avenue for exploring the impact of disruptions in adolescent social development on adult health. However, the impact of adolescent social experiences on adult physiology and behavior are complex and have led to inconsistent results. Inconsistency may result from many different factors including individual variability among genetically outbred rodents frequently used in these studies or outcomes may vary between the diverse social stress protocols used. As such, the goal of the first study was to determine whether individual differences in exploratory behavior modulate the impact of adolescent social experiences on adult HPA activity. We demonstrated the rats exhibiting high-exploratory behavior respond differently to exposure to novel social partners or individual housing during adolescence than do rats exhibiting low-exploratory behavior. Results suggest that individual differences in exploratory behavior (temperament) may modulate the influence of adolescent experiences on adult behavioral and adrenocortical function.

The results of the previous study suggest that individual differences in temperament contribute to inconsistent findings in the adolescent social stress literature. The cause of these individual differences is unclear, but could be due to genetic variability among the outbred rodents tested. Additionally, few studies have investigated sex differences in responses to adolescent social stress effects on adult behavior and physiology. The goal of the second study thesis study was to develop a novel adolescent social stress protocol which controlled for many potential sources of individual variability and systematically investigate sex-differences in the long-lasting effects of adolescent social stress. We used
this protocol to investigate stress effects on adult anxiety- and depression-related behavior and HPA axis activity. We sought to control for genetic variability by using an inbred mouse strain and individual differences in temperament by repeated exposure to both novel social partners and social isolation. We found that adolescent social stress increased anxiety and depression-related behavior and reduced adult HPA activity. We believe that this novel adolescent social stress protocol may be a valuable tool for future studies investigating the impact of disruptions to adolescent social development on adult behavior and physiology.

In the final study, we sought to address a novel research question with our social stress protocol. In humans, nicotine use and affective disorders are highly comorbid. Exposure to stress during adolescence is associated with increased risk for affective disorders and smoking initiation. These stress effects were attributed to elevated glucocorticoids. Stress, possibly due to glucocorticoid overexposure, can alter the development of stress-susceptible corticolimbic brain regions during adolescence. Thus, some common mechanism may underlie stress-related comorbidity of affective disorders and nicotine use. Rodent studies have indicated that adolescent social stress can increase anxiety and depression-related behavior, alter HPA activity, and influence nicotine responses. We sought to investigate whether stress-induced anxiety and depression-related behavior was related to altered nicotine responses in inbred mice using a within-subjects design. We found little evidence of a relationship between anxiety and depression-related behaviors and nicotine responses.
Taken together, the results of these experiments may help to clarify factors that contribute to inconsistent findings among adolescent social stress models and determine the impact of adolescent social stress on adrenocortical activity and comorbid affective disorders and nicotine dependence. Identification of the factors that promote maladaptive health trajectories during adolescence can help inform studies that elucidate the biological and environmental determinants that impinge on development of the HPA axis and brain regions that regulate reward- and affect-related behavior. These findings will inform future studies which can investigate these potential mechanisms.
1.9. References


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Chapter 2. Temperament Moderates the Influence of Periadolescent Social Experience on Behavior and Adrenocortical Activity in Adult Male Rats

2.1. Introduction

Adolescence is a unique period for social and adrenocortical development; it is a period when non-familial social experiences become more frequent while significant neuronal and adrenocortical maturation is occurring (Douglas et al., 2004; Romeo, 2010a; Spear, 2000). In particular, the acute glucocorticoid response in human and rodent adolescents requires a longer period to return to baseline levels compared to adults and prepubertal youth, and this may be an important developmental period for programming of adult adrenocortical responses (Foilb et al., 2011; Romeo, 2010a; Stroud et al., 2009; Walker, Walder, & Reynolds, 2001). Adaptations to current and future environments are also driven by adolescent social experiences and interact with glucocorticoid hormones to shape adult behavioral profiles (Sachser et al., 2013).

The rodent adolescent period is typically defined as postnatal day (PND) 28-46, with a characteristic increase in non-familial social interactions first evident at approximately PND 28 (Spear, 2000), and maturation of hypothalamic-pituitary-adrenal (HPA) axis negative feedback mechanisms throughout (Romeo, 2010a). Adolescent social experiences may shape adult behavior and HPA function via interactions between these two developmental phenomena.
Long-term effects of peripubertal experiences on behavioral and HPA axis profiles have been reported. For example, in rodents, adolescent chronic social stress (isolation, social reorganization, subordination during PND 28-70+) causes protracted corticosterone (CORT) responses and elevated basal CORT levels in adulthood and these effects can be prevented by anti-depressant (paroxetine) or corticotropin-releasing hormone 1 receptor antagonist (DMP696) administration during the stress procedure (Ros-Simó & Valverde, 2012; Schmidt et al., 2007; Sterleman et al., 2008; Toth, Mikics, Tulogdi, Aliczki, & Haller, 2011). However, models of adolescent stress are highly variable (e.g. social vs. physical stressor, novel social partners vs. social isolation, etc.), and outcomes of these models are inconsistent with numerous reports of unaffected adult adrenocortical activity after peripubertal stress (Isgor, Kabbaj, et al., 2004; Lukkes et al., 2009; McCormick et al., 2004, 2008). In the current study, we tested the hypothesis that long-term influences of adolescent experiences may be temperament-specific.

Behavioral inhibition (BI) or neophobia is a relatively stable behavioral trait in humans and rodents that emerges early in development (infancy) and is characterized by fear and avoidance of novel or unfamiliar situations and objects, and heightened cortisol reactivity after exposure to psychosocial stress, novel social situations, and novel objects (Cavigelli et al., 2007; Cavigelli & McClintock, 2003; Kagan, Reznick, & Snidman, 1987; Schmidt & Fox, 1997; Walker & Mason, 2012). In humans, this trait has been associated with increased risk of adolescent and adult mood disorders, which are often associated with altered adrenocortical regulation (e.g. Rosenbaum et al., 1993; Schwartz, Snidman, & Kagan, 1999). Incidentally, some studies (human and rodent) have also
shown greater glucocorticoid responses to novelty in non-inhibited (i.e. approach-oriented) individuals compared to inhibited ones (e.g. de Haan, Gunnar, Tout, Hart, & Stansbury, 1998; Dellu et al., 1996; Stansbury & Harris, 2000), and that behavioral responses to psychological stress that are incongruent with an individual’s preferred coping strategies may account for some of these seemingly contradictory findings (Stansbury & Harris, 2000; Tarullo, Mliner, & Gunnar, 2011). For example, in a novel peer interaction test children who exhibited behavior that was incongruent with their self-reported peer competence had larger HPA responses than children exhibiting congruent behavior, regardless of temperament (Stansbury & Harris, 2000). Furthermore, increased HPA activity was observed for highly inhibited children who were more social than those that were less social, and for uninhibited children who were less social than their uninhibited counterparts (Tarullo et al., 2011). Given increased fear-related responses to novel experiences, BI or neophobia may represent a specific trait that modulates how particular adolescent social experiences (novel social partners vs. lack of social partners) shape adult behavior and adrenocortical regulation. In the present study, we used a rodent model of neophobia to experimentally test the influence of several different adolescent social experiences (familiar kin partners, novel social partners, and no social partners) on the development of adult behavioral responses and glucocorticoid responses to novelty. We predicted that individuals that do not readily engage novelty (neophobic) will exhibit greater adrenocortical upregulation after complex, novel adolescent experiences (e.g. exposure to novel social partners) compared to after simple adolescent experiences (e.g. social isolation or familiar social partners). Additionally, we predicted that individuals that readily engage novelty (neophilic) will exhibit adrenocortical upregulation in
response to simple adolescent environments such as social isolation compared to more complex social experiences like exposure to novel partners.

Previously we found that rat (Sprague-Dawley) neophobia/philia, characterized by locomotion in an unfamiliar and protected arena, was related to latency to approach novelty, and was moderately stable from pre-weaning age throughout adulthood, and was reproducible across studies (Cavigelli et al., 2007; Cavigelli, Ragan, Michael, Kovacsics, & Bruske, 2009; Cavigelli & McClintock, 2003). Neophobic or inhibited males also had greater plasma CORT responses to novelty and stress compared with neophilic or non-inhibited males (Cavigelli et al., 2007; Cavigelli & McClintock, 2003; c.f. Dellu et al., 1996; Qi et al., 2010; Takahashi, 1992; Veenema, Sijtsma, Koolhaas, & de Kloet, 2005). Furthermore, neophobia is associated with decreased voluntary interactions with enriched environments and early environmental conditions can influence the development of this trait and its associated glucocorticoid profile (Tang, Reeb-Sutherland, Romeo, & McEwen, 2012). To our knowledge no one has assessed whether adolescent social experiences modify the development of this trait, and/or if this temperament dimension modifies the influence of adolescent social experiences on behavioral and glucocorticoid development into adulthood.

We tested an interactional ‘temperament x adolescent social experience’ hypothesis – i.e. that temperament modulates the influence of adolescent social challenges on adult behavioral and glucocorticoid response development. Specifically, we tested a ‘congruent-incongruent’ hypothesis that neophilic rats exposed to novel, complex social experiences would be less challenged by this ‘congruent’ adolescent
experience than neophobic rats exposed to the same novel complexity, and/or neophilic rats exposed to no social partners (i.e. ‘incongruent’ with their temperament), and that temperament-adolescent ‘incongruent’ experiences will lead to relatively long-term upregulation of glucocorticoid production. In addition, because previous work showed that temperamental traits observed in rodents and humans can be stable throughout life, we expected that locomotion in a novel environment would be relatively stable within individuals during the peripubertal and young adult periods, but that this behavioral trait may be modulated by adolescent social experiences.

2.2. Methods

2.2.1. Animals

Fifty-three male Sprague-Dawley rats from 15 litters were housed in solid-bottom plastic cages (43.5 x 23.5 x 20.5 cm). Rats were maintained on a 14L:10D lighting schedule with lights on at 2000 h (central standard time, CST) and ad libitum access to food and water. Cages were cleaned twice a week by trained animal facility personnel. The colony room was maintained at 22°C with ~50% humidity. All methods detailed below were approved by the University of Chicago Institute for Animal Care and Use Committee and adhered to the methods specified in the Guide for the Care and Use of Laboratory Animals (1996).

2.2.2. Overall Design

Rats were housed with the dam and littermates from birth (i.e. PND 0) until PND 22. At PND 18, each pup was given an individually-unique ear notch, and at PND 20
pups were tested on the Exploration Arena to estimate neophobia. To our knowledge, there are no studies evaluating the duration or magnitude of the effects of the ear notch procedure on subsequent locomotion, however, our personal observations of pups ear-notched at this age is that they return to original behavioral profiles within hours of the procedure. Rats were weaned at PND 22 and housed in same-sex sibling trios with similar temperament distribution in each cage (one neophobic rat, one neophilic rat, and one non-responsive rat – see ‘Exploration arena’ below). During PND 28-46, rats were placed in one of three experimental adolescent social conditions: (1) a control group (KIN) in which rats remained in groups of three same-sex littermates, (2) a social reorganization group (SRO) for which three unrelated same-sex novel social partners were housed together, or (3) an individual group (IND) in which rats were housed alone. In the KIN and SRO conditions, each group included one neophobic rat, one neophilic rat, and one non-responsive rat to ensure that social experiences were similar across all cages. These housing manipulations were developed to mimic social experiences that may be considered common during adolescent development in social species (e.g. moving to a new environment, social isolation, etc.) and were considered to be relatively short-lived and benign manipulations. On PND 46 all rats were rehoused in the original same-sex littermate trios.

To determine if adult exploratory behavior and/or glucocorticoid production were altered by these adolescent experiences and/or by a congruent-incongruent interaction between temperament and adolescent social experience, rats were again tested on the Exploration Arena at PND 60 and 85, and from PND 110-114 fecal samples were
collected and analyzed for fecal corticosteroid levels (see Glucocorticoid Measure below). To sample feces from individuals and to provide a complex novel challenge to stimulate glucocorticoid production, rats were placed in individual hanging cages on PND 110 (‘Day 1’) and then exposed to three novel social partners for one hour on PND 111 (‘Day 2’; see Social Challenge below). Fecal boli were regularly collected across all days.

2.2.3. Behavioral Response to Novelty

Behavioral testing was conducted in a non-colony room during the rat active period (4-6 hours after lights off). The room was illuminated with a red light providing approximately 6 lux of light at the center of the testing arena.

**Exploration arena.** This arena was used to assess rat exploratory behavior on PND 20, 40, 60, and 85. It was square with tall opaque walls (92 x 92 x 23 cm area for rats at PND 20; 122 x 122 x 46 cm for older rats) and a Plexiglas cover with a 3 x 3 grid that divided it into 9 equal divisions for quantification of locomotion. Inside the arena were three rat-sized objects that were placed 13 cm from each corner; objects were different for each test age to ensure object novelty. To provide familiar odors from cagemates and from colony room members, the floor was covered with clean wood chips that were then sprinkled with a small amount of bedding from all colony cages. If a rat defecated in the arena during testing, feces were removed and no further cleaning occurred. To begin the test the rat was placed in a ceramic bowl with 5 cm-high walls and lowered into the empty corner of the arena. The test lasted 5 minutes and each rat was video-recorded for
the duration of the test. At the conclusion each rat was returned to the home cage, transported back to the colony room, and then the test bowl rinsed with water and dried for the next rat. Locomotion in the arena was used to estimate neophobia/neophilia; locomotion was quantified as the number of times a rat crossed all 4 limbs over a line of the 3 x 3 grid on the arena cover. In prior studies, we have found that this measure of locomotion in the arena was closely related to latency to approach the novel objects in the arena at several different ages (Cavigelli et al., 2007). The locomotion score is a measure of behavioral activity in a novel environment, but it does not quantify which grid squares were visited or how much time was spent within each square.

Rat temperament (neophobic or neophilic) was assigned based on locomotion in the Exploration Arena at PND 20. Locomotion scores varied both between and within litters, thus to control for litter effects, we assigned temperament within litters, identifying 1-2 neophobic and 1-2 neophilic males from each litter. Within each litter, males with the highest locomotion scores were classified as neophilic and males with the lowest, non-zero locomotion scores as neophobic. The range of litter locomotion scores at PND 20 for neophobic vs. neophilic rats was 6-34 (for neophobic) and 33-54 (for neophilic). Rats that did not move in the arena were considered non-responsive and were not included in analyses.

2.2.4. Glucocorticoid Measures

**Fecal corticoids.** To assess basal and glucocorticoid responses to a complex, novel social experience we evaluated glucocorticoid diurnal rhythms in feces in response to individual
housing and a social challenge (see below). Fecal corticosteroid metabolite measures provide a minimally-invasive method to evaluate basal and long-term corticosterone production in response to complex and lengthy experimental manipulations while minimizing the influence of frequent blood sampling (Cavigelli et al., 2005). During the fecal collection period, rats were placed in cages with hanging wire bottoms through which feces dropped into a pan of standard wood-shaving bedding (Sani-Chips, Laboratory grade). Fecal samples were removed from the pan with forceps and placed in Whirl-pak bags (Nasco, Fort Atkinson, WI, USA), labeled, and stored at -30 °C until extraction. Forceps were cleaned with ethanol after each collection to avoid cross-contamination. Samples were collected at 3-hour intervals (600, 900, 1200, 1500, 1800, 2100, and 2400 hr) for 4 consecutive days. Given the ~6-12-hr lag time required for circulating steroids to be excreted in rat feces (Cavigelli et al., 2005; Harper & Austad, 2000), we were able to assess basal corticoid levels (from Day 1 samples), corticoid responses to novel individual-housing and novel social partners (from Days 2-3 samples), and recovery after novelty (Day 4 samples).

**Fecal corticoid extraction.** Fecal steroids were extracted using methods previously described (Cavigelli et al., 2005). Frozen samples were allowed to thaw and desiccated in a centrifugal evaporator and dry weights recorded. Dry samples were crushed into a fine powder, 0.2 g placed in a 15 ml polypropylene centrifuge tube and 10 ml of 100% ethanol added to each sample. Samples were boiled in a water bath for 20 min at 78°C, centrifuged at 2000g for 15 min, and the supernatant poured off into a borosilicate glass culture tube. Another 5 ml 100% ethanol was added to the fecal pellet and samples were
vortexed for 1 min, centrifuged for 15 min at 2000g, and the supernatant added to the previous 10 ml of extract. Samples were evaporated with air and reconstituted with 1 ml of methanol. After reconstitution all samples were stored at -80°C until assay. The outcome measure was total fecal corticoids excreted during each collection day. We used this total production measure to estimate total corticosterone production across a whole day as opposed to short-term responses to acute challenges (Cavigelli et al., 2005; Lepschy, Touma, & Palme, 2010). We also analyzed total dried fecal mass to determine if fecal mass was driving any of the total fecal corticoid results reported.

**Radioimmunoassay.** Fecal corticoid metabolites were measured using commercially-available $[^{125}I]$ Radioimmunoassay for rat and mouse serum/plasma (MP Biomedicals, Solon, OH). The antibody in this assay binds to 3 of 6 corticosterone metabolites in rats and thus provides a broad estimate of corticosterone production (Cavigelli et al., 2005; Thanos et al., 2009), and the fecal corticoid metabolite measure is closely related to circulating corticosterone levels. Samples were diluted 1:50 with the steroid diluent provided with the assay kit which ensured antibody binding on the linear portion of the standard curve (20-80% binding). The range of the standard curve was 12.5 to 1000 ng/ml. Duplicates were run for all samples and any sample with a coefficient of variation above 10% was re-analyzed. Inter-assay and intra-assay coefficients of variance were 10.7 and 8.6, respectively.

**Social challenge.** At 4 months of age rats were tested in a novel social situation. During a 1-hour social challenge, 3 rats that had no prior interactions with each other were placed in an arena with similar dimensions as the one used in the adult exploration arena tests.
The rats were of similar age and size. Each social challenge group included one neophobic rat, one neophilic rat, and one non-responsive rat. After the social challenge rats were placed in their wire-bottom home cages and returned to the colony room.

2.2.5. Statistical Analyses

Prior to statistical analyses we tested whether data were normally-distributed (according to Kolmogorov-Smirnov tests). Because fecal corticoid output for days 2 through 4 were not normally-distributed, we used log-transformed values to satisfy distribution requirements for parametric statistical analyses. For clarity, data are presented as untransformed values in figures and the table. All analyses were conducted with SPSS version 22.

To test the hypothesis that individual temperament status was stable over time, repeatability of locomotor behavior in the test arena was estimated from an intraclass correlation coefficient (ICC) using locomotion scores across the 4 test ages (PND 20, 40, 60, 85). The ICC is based on variance components derived from a one-way analysis of variance (ANOVA) and is calculated as \( r = \frac{s_{a}^2}{s^2 + s_{a}^2} \), where \( s^2 \) is within-individual variance overtime and \( s_{a}^2 \) is the among-litter variance (Lessells & Boag, 1987). ICC is often used to determine individual consistency in repeatedly measured behaviors both in the field and laboratory (Bell, Hankison, & Laskowski, 2009; Lessells & Boag, 1987). To determine if adult exploratory behavior was affected by weanling exploratory behavior, adolescent social conditions, and/or an interaction between weanling temperament and adolescent experiences, a 2 x 3 (temperament x adolescent condition) ANOVA was used.
with adult (PND 60 and 85) locomotion values as dependent variables (n=53). To verify that weanling locomotor behavior did not differ among the adolescent experimental groups, a similar ANOVA was conducted PND 20 locomotion values as the dependent variable (n=53). To estimate effect sizes for all ANOVA main and interaction effects, eta squared ($\eta^2$) was calculated to indicate the proportion of variance in the dependent variable that was accounted for by the independent variable. Cohen’s $d$ was used to estimate effect size for pairwise comparisons. Because locomotion varied both within and between litters (e.g., the range of locomotion scores for two extreme litters were 22-79 vs. 69-117), litter mean locomotion scores were included as a covariates in the above models to control for between-litter variance.

To compare daily fecal corticoid production across sampling days, a repeated measures ANOVA was used with time as the within subjects factor. To assess the effects of temperament and adolescent social conditions on adult baseline fecal corticoid output, a $2 \times 3$ (temperament $\times$ adolescent condition) ANOVA was conducted using Day 1 fecal measures (n=45; not all males defecated during this baseline period). To determine if adult glucocorticoid responses to novelty were affected by temperament, adolescent social conditions or an interaction between these factors, two $2 \times 3$ (temperament $\times$ adolescent condition) ANOVAs were conducted with total corticoid metabolite production and total daily fecal mass production on the second and third days of fecal collection, when fecal corticoid levels were highest (n=53). Last, to determine if weanling temperament and/or adolescent social conditions affected adult rates of glucocorticoid recovery (i.e. the ability to return to basal levels), two similar ANOVAs
were conducted with total fecal corticoid levels and fecal mass on the last day of sampling – the fourth day after adult individual housing began (n=53). Total daily fecal mass production was assessed to ensure that differences in fecal corticoid levels were not due to differences in the amount of fecal mass produced between groups. Total dry fecal mass was not included in the final model because it did not significantly affect fecal corticoid levels. All post-hoc analyses were conducted with a Bonferroni correction for multiple comparisons, \( p < 0.05 \).

Daily total fecal corticoid production on days 2, 3, and 4 were calculated as the sum total (ng) of fecal corticoids produced from 900-900 hr the following day. Mean total daily fecal corticoid production varied considerably between and within litters, thus mean litter production was used as a covariate in the above models. Eight rats did not defecate during day 1 of individual housing: 2 neophilic males (1 KIN, 1 SRO), and 6 neophobic males (1 KIN, 4 IND, 1 SRO). Independent-samples t-tests were conducted to compare behavior in the exploration arena and daily fecal corticoid output between the males that were removed from analyses and those that were not removed. Males that did not defecate on day 1 had significantly lower locomotion in the exploration arena on PND 20 than males that did defecate on day 1 (\( \bar{X} \pm SD: 21 \pm 19 \) vs. \( 36 \pm 17 \), \( t_{51} = 2.28, p < 0.05 \), Cohen’s \( d = 0.63 \)). There were no other differences between males that did and did not defecate on day 1 in behavior or in total fecal corticoid output over days 2-4.
2.3. Results

2.3.1. Weanling and Adult Behavioral Response to Novelty

An intraclass correlational analysis revealed that locomotion in a novel, complex environment was relatively stable (i.e. repeatable) within individuals across PND 20, 40, 60, and 85 ($r_{49,147} = 0.75$, $p > 0.05$). There were no main effects of weanling-based temperament or adolescent social condition on young adult (PND 60) locomotion in the exploration arena ($F_{1,51} = 0.16$, $p = 0.70$, $\eta^2 = 0.00$; $F_{2,51} = 0.51$, $p = 0.60$, $\eta^2 = 0.00$), but there was an interaction effect of temperament and adolescent condition ($F_{2,51} = 4.33$, $p < 0.05$, $\eta^2 = 0.05$; Figure 2-1A). Interestingly, this interaction effect was no longer present at PND 85 ($F_{2,52} = 0.67$, $p = 0.52$, $\eta^2 = 0.01$), and there was only a main effect of weanling-based temperament on adult (PND 85) locomotion in the exploration arena: males identified as neophilic at weaning crossed significantly more lines as adults in the exploration arena than did males identified as neophobic at weaning ($F_{1,52} = 4.64$, $p < 0.05$, $\eta^2 = 0.05$; Figure 2-1B). At this later age, there were no significant differences in locomotion among the three adolescent social conditions ($F_{2,52} = 1.67$, $p = 0.20$, $\eta^2 = 0.03$). Weanling locomotion on PND 20 did not differ among the KIN, SRO, and IND males ($\bar{X} \pm \text{S.E.M.}: 34 \pm 2$ vs. $35 \pm 2$ vs. $33 \pm 2$; $F_{2,52} = 0.19$, $p = 0.83$, $\eta^2 = 0.00$) nor was
there an interaction effect of temperament and adolescent condition on locomotion at this age ($F_{2,52} = 1.51, p = 0.23, \eta^2 = 0.02$).

Figure 2-1. Estimated marginal means for locomotion for 53 male rats during 5-minute trials in the exploration arena. (A) * On postnatal day (PND) 60, males identified as neophilic at weaning and that experienced adolescent social reorganization (SRO) crossed significantly more lines than neophobic males that experienced SRO. † Conversely, neophobic males that remained with siblings during adolescence (KIN) crossed significantly more lines than neophilic KIN males. (B) ‡ On PND 85, males identified as neophobic at weaning crossed significantly fewer lines than did neophilic males (all $p$’s < 0.05). Error bars indicate S.E.M.

2.3.2. Adult fecal corticoids

Adult males displayed a circadian rhythm in fecal corticoid production across the four consecutive sampling days, with a significant effect of day on total daily fecal corticoid production ($F_{1,52} = 4.24, p < 0.05, \eta^2 = 0.08$): males produced significantly more fecal corticoids on Day 3 than on Day 4 (Figure 2-2).
Baseline. Adolescent social conditions had a significant effect on adult basal fecal corticoid production (on ‘Day 1’ or PND 110; $F_{2,44}= 4.83, p < 0.05, \eta^2 = 0.07$). KIN males had significantly higher baseline fecal corticoid output than SRO males, and IND males did not differ from KIN or SRO males (Figure 2-3). Weanling temperament tended to predict mean baseline fecal corticoid production, but this effect did not reach statistical significance ($F_{1,44}=3.83, p < 0.10, \eta^2 = 0.03$), and there was no interaction between temperament and adolescent condition ($F_{2,44}= 0.68, p = 0.51, \eta^2 = 0.00$). Total dry fecal mass produced during day 1 did not differ between neophobic and neophilic males ($\bar{X} \pm \text{SEM}$).
S.E.M.: 1.75 ± 0.44 vs. 1.58 ± 0.14; $F_{2,44} = 0.62$, $p = 0.44$, $\eta^2 = 0.01$), nor among KIN, SRO, and IND males ($\bar{X} \pm \text{S.E.M.}: 1.89 \pm 0.17$ vs. 1.71 ± 0.18 vs. 1.40 ± 0.19; $F_{2,44} = 1.83$, $p = 0.17$, $\eta^2 = 0.05$), and there was no interaction of temperament and adolescent condition ($F_{2,44} = 1.70$, $p = 0.20$, $\eta^2 = 0.05$).

**Social Challenge.** Neither weanling temperament ($F_{1,52} = 1.50$, $p = 0.23$, $\eta^2 = 0.03$) nor adolescent social condition ($F_{2,52} = 1.03$, $p = 0.37$, $\eta^2 = 0.03$) affected day 2 fecal corticoid production, nor was there an interaction ($F_{2,52} = 0.15$, $p = 0.87$, $\eta^2 = 0.00$). Adolescent

![Figure 2-3](image-url)

Figure 2-3. Estimated marginal means for basal fecal corticoid production prior to adult individual housing and social challenge (day 1). * Males that remained with siblings in adolescence (KIN) males had significantly higher basal fecal corticoid output than social reorganization (SRO) males, $p < 0.05$. Error bars indicate S.E.M.
social condition affected day 3 fecal corticoid production with KIN males producing more fecal corticoids compared to SRO and IND males ($F_{2,52} = 3.26, p < 0.05, \eta^2 = 0.08$; Table 2-1), however day 3 fecal corticoid production did not differ between neophobic and neophilic males ($F_{1,52} = 0.15, p = 0.70, \eta^2 = 0.00$), and there was no interaction between weanling temperament and adolescent social condition ($F_{2,52} = 0.13, p = 0.88, \eta^2 = 0.00$). Fecal corticoid recovery levels (day 4) were significantly affected by adolescent social condition with KIN males producing significantly more fecal corticoids than SRO males ($F_{2,52} = 4.15, p < 0.05, \eta^2 = 0.06$). In relation to the congruent-incongruent hypothesis, there was a significant interaction between weanling temperament and adolescent social condition ($F_{2,52} = 4.67, p < 0.05, \eta^2 = 0.07$; Figure 2-4); neophilic males that had experienced novel social partners during adolescence (SRO) produced less fecal corticoids than neophobic males that had this same adolescent experience (SRO) and neophilic males that experienced no new social partners during adolescence (IND, KIN). Further, individual-housing negated the difference in fecal corticoid production between neophobic and neophilic males. There was no main effect of weanling-based temperament on day 4 fecal corticoid recovery ($F_{1,52} = 0.97, p = 0.33, \eta^2 = 0.00$). Total dry fecal mass produced on each day did not differ between weanling-based temperament categories ($\bar{X} \pm \text{S.E.M.}$ for days 2, 3, 4: neophobic = 4.70 ± 0.20, 5.13 ± 0.30, 5.70± 0.22, neophilic = 4.86 ± 0.20, 5.14 ± 0.32, 5.38 ± 0.22; $F_{1,52} = 0.40, p = 0.53, \eta^2 = 0.00$, $F_{1,52} = 0.00, p = 0.99, \eta^2 = 0.00, F_{1,52} = 1.03, p = 0.32, \eta^2 = 0.02$) or among adolescent social conditions ($\bar{X} \pm \text{S.E.M.}$ Days 2, 3, 4: KIN = 4.74 ± 0.24, 5.31 ± 0.38, 5.81 ± 0.27, SRO = 4.82 ± 0.25, 4.41 ± 0.40, 5.03 ± 0.28, IND = 4.76 ± 0.24, 5.69 ± 0.38, 5.79 ± 0.27; $F_{2,52} = 0.03, p = 0.97, \eta^2 = 0.00, F_{2,52} = 2.78, p = 0.07, \eta^2 = 0.09, F_{2,52} = 2.56, p = 0.09, \eta^2 =$}
0.08). There were no interactions of temperament and adolescent social condition for
days 2, 3, or 4 (F_{2,52} = 0.08, p = 0.92, \eta^2 = 0.00; F_{2,52} = 0.48, p = 0.62, \eta^2 = 0.02; F_{2,52} =
0.64, p = 0.53, \eta^2 = 0.02).
Table 2-1. Mean (S.E.M) total daily fecal corticoid production (ng) in response to individual housing and adult social challenge.

<table>
<thead>
<tr>
<th>Time (day):</th>
<th>Pubertal condition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KIN ((n = 18))</td>
</tr>
<tr>
<td>Day 2</td>
<td>2562 (217)</td>
</tr>
<tr>
<td>Day 3</td>
<td>2930 (229)(^a)</td>
</tr>
</tbody>
</table>

*Note. KIN = control; SRO = social reorganization; IND = individual housing.

\(^a\) Day 3: KIN > SRO and IND, \(p < 0.05\).

Figure 2-4. Estimated marginal means for total fecal corticoid production during recovery from adult individual housing and social challenge (day 4). * Males that experienced adolescent social reorganization (SRO) had significantly lower total fecal corticoid production than males that remained with siblings (KIN). † Males that were identified as neophilic at weaning and exposed to SRO during adolescence had lower total fecal corticoid production than neophobic males that experienced SRO. This temperament-specific corticoid production was not evident in the males that had experienced KIN or individual housing (IND) during adolescence. (All \(p\)'s < 0.05). Error bars indicate S.E.M.
2.4. Discussion

2.4.1. Temperament/Exploratory Behavior

The results of the present study replicate previous findings that male Sprague-Dawley rats, like human children, display relatively stable individual differences in exploratory behavioral responses to novelty, with weanling exploratory responses predicting adult exploratory behavior (Cavigelli et al., 2007, 2009; Cavigelli & McClintock, 2003). Furthermore, the results indicate that these traits were relatively stable from weaning to young adulthood, even after experiencing divergent adolescent social experiences (i.e. familiar social partners vs. novel social partners vs. social isolation). However, there was evidence of a short-term alteration in exploratory behavior at PND 60 that reflected an interaction of temperament with adolescent social experiences. Specifically, neophobic weanling rats that were housed with novel social partners (SRO) during adolescence (i.e. a hypothesized incongruent social experience relative to temperament) showed decreased exploratory behavior at PND 60 compared to neophobic rats that had been housed with familiar littermates (KIN) or alone (IND) during adolescence (i.e. temperament-congruent social experiences; Figure 2-1A). On the other hand, neophilic weanling rats exposed to novel social partners during adolescence (i.e. temperament-congruent social experience) showed increased exploratory behavior at PND 60 compared to neophilic rats that were housed with familiar social partners and/or alone during adolescence (i.e. hypothesized temperament-incongruent conditions). Although this interaction of temperament and adolescent social experience was evident at PND 60, this effect dissipated by PND 85 (Figure 2-1B).
The tendency to approach or avoid novel objects and people, and the amount of interaction with novel stimuli, are frequently-used method for classifying a child as non-inhibited or inhibited (Kagan et al., 1987); similar methods can be used to classify animals as non-inhibited or inhibited. As in humans, animal behavioral inhibition tends to be associated with elevated glucocorticoid production, and is believed to be highly conserved across species (Cavigelli, 2005; Cavigelli et al., 2007; Gosling & John, 1999; Roseboom et al., 2007; Schulkin, Gold, & McEwen, 1998; Takahashi, 1994). These results indicate that naturally-occurring individual differences in exploratory behavior in outbred rodents provide a viable model of human behavioral inhibition.

Recent evidence suggests that the developmental stability of temperament is not as stable as once thought. Environmental factors, in particular maternal factors, may influence the plasticity of temperament (Tang et al., 2012). However, non-maternal social influences may also affect temperament development. For example, in rats the ratio of males to females in a litter predicts 4-month-old, although not 3-month-old, locomotion in an open field (Gracceva, Koolhaas, & Groothuis, 2011). Furthermore, the stability of personality increases significantly during the transition from adolescence to young adulthood in humans (Roberts, Caspi, & Moffitt, 2001). The results from the current study indicate that ‘congruent’ vs. ‘incongruent’ social experiences during adolescence may lead to short-term alterations in exploratory behavior, but that original levels of exploration re-emerge over time. Similar results have been shown in orange-winged Amazon parrots: birds that were hand-reared by humans as neonates showed transient reductions in neophobic behavior, compared to birds that were reared by parents, and that
this change disappeared in adulthood (Fox & Millam, 2004). Prior studies have suggested greater behavioral plasticity during infancy and adolescence as compared to adulthood, although this area requires further experimental work (Moretz, Martins, & Robison, 2007; Roberts et al., 2001). Thus, infancy and adolescence may represent a period of plasticity in the development of temperament, with short-term alterations in social conditions leading to transient changes in behavior. Future studies will need to determine if longer-term (congruent or incongruent) social experiences during these early developmental periods lead to long-term alterations in temperament.

2.4.2. Adult Glucocorticoid Production

A characteristic circadian rhythm is evident in adult male fecal corticoid production (Figure 2-2). Similar results have been observed in prior studies and these results indicate that fecal corticoid production can accurately portray daily fluctuations in adrenal activity as well as adrenal response to an environmental challenge (Cavigelli et al., 2005). At baseline, there was a trend for neophobic males to produce more fecal corticoids, although this difference was not statistically significant. These results differ from prior studies in rats that used the same behavioral protocol and circulating corticosterone measures and in humans in which BI children, teens, and adults showed elevated afternoon cortisol and enhanced cortisol reactivity (Cavigelli et al., 2007; Cavigelli & McClintock, 2003; Essex, Klein, Slattery, Goldsmith, & Kalin, 2010; Kagan et al., 1987; Schmidt & Fox, 1997; Tyrka, Wier, Price, & Rikhye, 2008). In the current study, there was a significant bias in the number of males that produced feces during this period, with the neophobic males producing feces less often than neophilic males, which
may have led to both decreased statistical power and a sampling bias. Interestingly, stable BI is associated with chronic constipation in human children (Reznick et al., 1986). Additionally, daily integrated measures of fecal corticoid production may not be sensitive enough to detect small, albeit likely functionally-significant, differences in glucocorticoid production between inhibited and non-inhibited individuals (Thanos et al., 2009).

Temperament differences in circulating glucocorticoids must be substantial and persist for several hours in order to be detected in fecal samples (Siswanto, Hau, Carlsson, Goldkuhl, & Abelson, 2008).

Social experiences in adolescence shape adult behavioral and neuroendocrine responses (Sachser et al., 2013). In the current study, two-weeks of an adolescent social experience led to significant alterations in adult corticoid excretion at baseline and during exposure to social novelty, and these effects persisted longer than observed alterations in behavior. Specifically, rats that lived with novel social partners during adolescence (SRO) produced less fecal corticoids at baseline and in response to individual-housing and brief exposure to novel social partners in adulthood compared to rats that continuously lived with familiar siblings during adolescence (KIN; Table 2-1, Figures 2-3 & 2-4). This alteration in adult glucocorticoid production was documented more than 2-months following the adolescent social experience suggesting that there was a long-term recalibration of adrenocortical activity. Similar findings of adult hypothalamic-pituitary-adrenal (HPA) hyporeactivity have been documented in humans and rodents that experienced more severe physical and social stressors during adolescence or adulthood (e.g., four to six weeks of exposure to immobilization, change of cage mate, cage tilt,
exposure to white noise, exposure to predator odor; Bazak et al., 2009; Engert et al., 2010; Goliszek et al., 1996; Ostrander, Ulrich-Lai, Choi, Richtand, & Herman, 2006; Ros-Simó & Valverde, 2012; Schmidt et al., 2007; Toth et al., 2008). Fries, Hesse, Hellhammer, and Hellhammer (2005) proposed that chronic stress exposure may lead to an initial period of glucocorticoid hypersecretion followed by subsequent HPA hypoactivity. With regard to this theory, adolescent experiences may prepare an organism for its future social environment and regulate HPA axis activity accordingly (Sachser et al., 2013). Hypoactive HPA activity may be an adaptation to prevent negative effects of prolonged stress hormone exposure. However, an alternative interpretation of these results is possible. SRO males may not have produced as much corticosterone as KIN males in response to the adult novel social challenge because it was not as novel an experience; SRO males had already experienced similar social novelty during adolescence which may have led to habituation of the HPA response. Future research would be necessary to determine whether adolescent social experience causes short-term HPA hyperreactivity followed by hypoactivity postulated in Fries and colleagues’ theory of the development of hypocortisolism (Fries, Hesse, Hellhammer, & Hellhammer, 2005).

The last result from the current study indicates that stable inter-individual differences in behavioral responses to novelty may moderate the influence of adolescent social experiences on adult adrenocortical activity. Specifically, neophilic males that were exposed to novel social partners during adolescence showed significantly shorter increase in adult glucocorticoid production after a complex social challenge compared to
neophilic males that received no novel social stimulation during adolescence (SRO vs KIN/IND; Figure 2-4). This is comparable to a handful of studies which indicate that children that behave in a manner that is congruent with their preferred behavioral coping strategies (e.g., approach or avoid novel or challenging situations) exhibited lower cortisol responses than those that behaved in an incongruent fashion (Stansbury & Harris, 2000; Tarullo et al., 2011). Of note, these children chose to approach or avoid social interaction in these studies, whereas in the current experiment, rats were assigned to a particular adolescent social condition. These prior findings in children, in conjunction with the current longer-term study with rodents, suggest that temperament may modulate the degree to which adolescent social experiences have long-term impacts on adrenocortical function.

Genetics and early life experiences are additional factors that may influence the development of temperament over and above the effects of adolescent social experiences. BI is moderately heritable (Oler et al., 2010; Smoller et al., 2005). In humans, genetic markers associated with BI include single nucleotide polymorphisms (SNPs) in the gene encoding corticotropin releasing hormone (CRH) which regulates fear behavior and HPA axis activation (Smoller et al., 2005). In a non-human primate model of BI, SNPs in the CRH receptor 1 gene and metabolic activity in the hippocampus, which mediates inhibition of the HPA axis stress response via negative feedback, in response to social novelty were heritable and predicted BI (Oler et al., 2010; Rogers et al., 2013). Maternal caretaking also influences behavior and HPA axis activity via epigenetic modifications of HPA axis regulatory genes (Weaver et al., 2004). In the current study, we controlled for
covariation in exploratory behavior and HPA axis activity between litters. Future research is necessary to determine the effect of these early life experiences and genetic factors on the development of temperament and HPA axis activity.

2.4.3. Conclusion

Stable individual differences in rodent exploratory temperament were established early in life based on methods that were comparable to those used to classify childhood behavioral inhibition in humans. Rats that exhibited low-exploration at weaning also exhibited low-exploration in adulthood, although this behavioral disposition was temporarily shifted depending on adolescent social experiences. Furthermore, a short-term novel social experience in adolescence, as compared to stable social housing with littermates/kin, was associated with a significant decrease in adult basal glucocorticoid secretion and a tendency towards low total daily glucocorticoid production after social novelty. Finally, return to basal glucocorticoid production was more rapid in rats that experienced adolescent social conditions that were congruent with their weanling-established temperament. Specifically, neophilic rats that experienced novel social partners during adolescence (i.e., a temperament-congruent social experience) had lower adult glucocorticoid production four days into a novel social experience than neophilic rats that had no novel social partners during adolescence and/or neophobic rats that were exposed to social partners during adolescence (i.e., temperament-incongruent social experiences). In sum, relatively short-term adolescent social experiences can lead to transient changes in temperament and longer-lasting changes in HPA axis regulation, with the specific influence of adolescent social experiences on HPA axis function
potentially moderated by early temperament and its relative congruency/incongruency with the adolescent social experience. Interestingly, some long-term physiological consequences of early life experiences may persist despite a lack of persistently altered behavior.
2.5. References


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Chapter 3. Exposure to chronic variable social stress during adolescence alters affect-related behaviors and adrenocortical activity in adult male and female inbred mice

3.1. Introduction

Adolescence is a developmental period characterized by profound behavioral and neurobiological changes associated with increased novelty seeking and emotionality (Brenhouse & Andersen, 2011; Spear, 2000). Peer-directed social interactions are particularly salient to adolescents as they fine-tune complex social skills (Sachser et al., 2013). Human studies suggest that the adolescent brain may be uniquely susceptible to long-lasting stress effects that can manifest as affective disorders into adulthood (Heim & Nemeroff, 2001). Preclinical studies also suggest that adolescents are vulnerable to social stress; social isolation, social defeat, and social instability all increase adult anxiety- and depression-related behaviors in rats and mice (Bourke & Neigh, 2011; Hong et al., 2012; Lukkes et al., 2009; Ros-Simó & Valverde, 2012; Scharf et al., 2013; Schmidt, Scharf, Liebl, et al., 2010; Schmidt, Trümbach, et al., 2010; Vidal et al., 2007). These social stress paradigms have primarily been implemented in outbred male rodents. This presents a limitation because genetic manipulations – powerful tools to elucidate biological mechanisms of complex phenotypes – are normally generated in inbred mouse strains and results are highly dependent on genetic background (Sittig et al., 2016). Furthermore, few studies have specifically compared the effects of adolescent social stress in both sexes and on both anxiety- and depression-related behavior (Table 1). Given these limitations, the current study measured adult anxiety- and depression-related behavior in male and female inbred mice exposed to a novel adolescent social stress protocol. Our protocol
included stressors that are known to affect both female and male mice (i.e. social isolation and novelty, respectively) (Arndt et al., 2009; Bartolomucci et al., 2004; Brown & Grunberg, 1995).

Table 3-1. Experiments investigating the impact of adolescent social stress on adult affective-like behavior.

<table>
<thead>
<tr>
<th>Author</th>
<th>Sex</th>
<th>Strain + Species</th>
<th>Age at stress:</th>
<th>Stress type:</th>
<th>Age at testing:</th>
<th>Test:</th>
<th>Anxiety and depression:</th>
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<tr>
<td>Schmidt et al. (2010a)</td>
<td>Female</td>
<td>CD1 Mice</td>
<td>P31 - P80</td>
<td>*Social Instability</td>
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<td>NSFT</td>
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<td>Schmidt et al. (2010b)</td>
<td>Male</td>
<td>CD1 Mice - Resilient</td>
<td>P31 - P80</td>
<td>*Social Instability</td>
<td>P108</td>
<td>OFT</td>
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<td>CD1 Mice - Vulnerable</td>
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<td>Scharf et al. (2013)</td>
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<td>CD1 Mice</td>
<td>P28 - P77</td>
<td>*Social Instability</td>
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<td>McCormick et al. (2008)</td>
<td>Male</td>
<td>Long-Evans Rats</td>
<td>P30 - P45</td>
<td>*Social Instability</td>
<td>P70</td>
<td>EPM</td>
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<td></td>
<td>Female</td>
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<td>Bourke &amp; Neigh (2011)</td>
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<td>Wistar Rats</td>
<td>P37 - P49</td>
<td>Mixed Modality</td>
<td>P96</td>
<td>EPM</td>
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<td>Wistar Rats</td>
<td>P45 - P47</td>
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<td>SA</td>
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<td>CD1 Mice</td>
<td>P21 - P70</td>
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<td>P21 - P42</td>
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Note: NSFT = Novelty suppressed feeding test; OFT = Open field test; SI = Social interaction test; EPM = Elevated plus-maze; TST = Tail suspension test; SPT = Sucrose preference test; SA = Social Avoidance; LDB = Light-Dark Box; *Cage composition changed 2x/week for 7 weeks; †1 h daily social isolation + re-housing with a novel cagemates; ‡ Increased depression/anxiety-related behavior; ↓ No change in behavior;
In rodents, adolescent development is typically considered to occur from postnatal day (PND) 21–59 (McCormick & Mathews, 2010). During this time, the hypothalamic-pituitary-adrenal axis (HPA) undergoes significant maturation, which can be modified by chronic stress. For example, adolescent social stress leads to blunted basal corticosterone levels in adult mice and rats which may follow an initial period of HPA hyperactivity during stress exposure (Caruso, McClintock, & Cavigelli, 2014; Ros-Simó & Valverde, 2012; Scharf et al., 2013; Schmidt, Scharf, Liebl, et al., 2010). Stress-induced alterations in HPA regulation are one way in which adolescent stress may increase adult affective disorders (McCormick & Green, 2013). Prolonged glucocorticoid (GC) exposure due to repeated stress may alter adolescent hippocampus, amygdala, and prefrontal cortex development resulting in sensitization of stress-responsive neural circuits that regulate emotion-related behavior (Heim & Nemeroff, 2001; Isgor, Kabbaj, et al., 2004; Leussis & Andersen, 2008; McCormick, Hodges, & Simone, 2015b). Adolescent social stress leads to reductions in myelination, synaptic density, and dendritic complexity in these regions (Leussis & Andersen, 2008; McCormick & Green, 2013; Tsai et al., 2014), although the precise mechanisms by which chronic adolescent social stress leads to anxiety and depression symptoms remain unclear. Rodent models are important tools for investigating these processes. However, the results of adolescent social stress studies in rodents are quite variable (Table 1). Several studies report significant sex-differences in vulnerability, while others report no enduring neurobiological or behavioral changes in either sex (Bourke & Neigh, 2011; Hong et al., 2012; Isgor et al., 2004). Different outcomes among studies may be compounded by genetic variability in commonly-studied
outbred animals (e.g. Sprague-Dawley rats, Long-Evans rats, and CD1 mice) (Caruso, McClintock, & Cavigelli, 2014; Scharf et al., 2013; Schmidt, Trümbach, et al., 2010).

The early adolescent stage is a sensitive period in rodent development during which social stress (e.g. isolation or social instability) can cause persistent alterations in adult behavior (McCormick et al., 2015b; Sachser et al., 1998). Research with inbred mouse strains can facilitate the discovery of genetic and environmental factors mediating the adverse effects of adolescent social stress (Jacobson & Cryan, 2007). However, limitations of established social stress models should be taken into account for research involving inbred mouse strains. Paradigms utilizing social instability (e.g., repeat exposure to novel social partners) are advantageous because large cohorts of mice can be exposed to uncontrollable and continuously stressful situations. Social instability disrupts social group composition and increases inter-male aggression during a time when social skills are acquired (Sachser et al., 2013; Schmidt et al., 2007; Schmidt, Scharf, Liebl, et al., 2010). In addition, different inbred mouse strains show different levels of baseline sociability (e.g. Brodkin 2007), and thus strain selection may influence outcomes. Isolation is also easily applied, but may be more stressful for females than males or for high- vs. low-sociability inbred mice (e.g. C57BL/6J vs. BALB/cJ) (Arndt et al., 2009; Bartolomucci et al., 2004; Brown & Grunberg, 1995). For example, isolation increased basal corticosterone levels in adult female C57BL/6NCrl mice but had no effect on female BALB/cAnNCrl mice or males of either strain (Arndt et al., 2009). Finally, adolescent social stress exposure should be limited to developmental periods within an age-range that is commonly-accepted as rodent adolescence (PND 21-59), but some
researchers utilize stress protocols that continue into adulthood (Table 1). Thus, the chronic variable social stress (CVSS) protocol was designed to be ethologically-appropriate, age-specific, and to include multiple adolescent social stressors that may affect both males and females across multiple inbred mouse strains.

The current study tested the effects of CVSS, administered during adolescent development, on adult behavior and HPA function in inbred BALB/cJ male and female mice. BALB/cJ mice were chosen for this initial experiment because they are typically considered to be a high-anxiety strain and a number of studies have demonstrated that they are more stress-susceptible than other inbred strains (reviewed in Belzung & Griebel, 2001; Jacobson & Cryan, 2007). The combined effect of repeated adolescent social isolation and novelty exposure on adult HPA activity and behavior in both male and female inbred mice remains uncharacterized. We hypothesized that adolescent CVSS would increase adult anxiety- and depression-related behaviors and alter basal HPA activity in both sexes.

3.2. Methods

3.2.1. Animals

Male and female BALB/cJ mice (The Jackson Laboratory, Bar Harbor, ME) were bred and pups remained with the dam until weaning (PND 22). Mice were housed in polycarbonate cages (28 cm x 17 cm x 12 cm) with corn-cob bedding and maintained on a reverse 12:12 h light:dark schedule (lights off 06:00 h) with ad libitum food and water. All procedures were approved by the Pennsylvania State University IACUC committee.
3.2.2. **Chronic Variable Social Stress Procedure**

At weaning, mice from 8 litters were assigned to either CVSS or control (CON) groups ($n = 7-9$/sex/group), with littermates evenly distributed among groups. CVSS was administered from PND 22-50. This age-range was chosen to limit stress exposure to a developmental period commonly accepted as the early- to late-adolescent stages of development, based on age-specific behavioral, neurobiological, and pubertal changes (McCormick & Mathews, 2010). Note that early adolescence has also been designated as the juvenile period (Andersen, 2003; Spear, 2000). Timing and duration of CVSS were consistent with previous adolescent social stress protocols used with outbred mice, with the exception that CVSS concluded during late-adolescence, whereas some prior stress protocols have continued into adulthood (Scharf et al., 2013; M. V. Schmidt, Scharf, Liebl, et al., 2010; M. V. Schmidt, Trümbach, et al., 2010). CVSS consisted of repeated cycles of individual-housing (3 days) and re-socialization with 2 unfamiliar same-sex cagemates (4 days). CON mice were housed with same-sex cagemates (3/cage) where they remained throughout adolescence.

3.2.3. **Glucocorticoid Measurements**

CVSS effects on HPA activity were assessed with non-invasive measures of fecal corticoid metabolites (fCORT) to estimate diurnal peak and trough corticosterone production (Cavigelli et al., 2005). Measures of plasma corticosterone more precisely reflect stress-induced changes in HPA activity, but repeated blood sampling can cause additional stress and elevate corticosterone levels within minutes (Touma, Sachser,
Möstl, & Palme, 2003; Tuli, Smith, & Morton, 1995). Fecal measures limit the impact of repeated sampling procedures on subsequent behavior and circadian corticosterone rhythms to provide an integrated measure of unbound (i.e., biologically active) corticosterone (Cavigelli et al., 2005). On PND 51, all mice were moved from group- to individual-housing in wire-bottom cages for 1-week of acclimation prior to fecal collection. We have previously found that basal fCORT levels return to pre-isolation levels after 4 days (Caruso et al., 2014). On PND 59, feces were collected every 4h, with two time points during the diurnal fCORT peak (08:00 and 12:00 h) and two times during the trough (20:00 and 24:00 h). fCORT was extracted then quantified using radioimmunoassay [125]I kits (MP Biomedicals, Solon, OH) as previously described (Caruso et al., 2014). Briefly, fCORT was extracted with ethanol, evaporated with air, and reconstituted in methanol. Reconstituted samples were diluted with kit-provided diluent (1:5) to ensure primary binding along the linear portion of the standard curve (20-80% binding). Samples were assayed in duplicate and those with a coefficient of variation greater than 15% were re-analyzed. Inter-assay and intra-assay coefficients of variance were 10.3 and 9.7%, respectively. We analyzed mean peak and trough fCORT concentrations (ng/g) and total dried fecal mass (g). Following fecal collection, mice were re-housed with their original same-sex cagemates (3/cage) on regular bedding.

3.2.4. Behavioral Testing

Elevated Plus-Maze. On PND 60, anxiety-related behavior was measured on the elevated plus-maze (EPM) under dim red lights (~30-50 lux) during the active period (13:00 – 17:00 h). Consistent with prior studies in the adolescent social stress literature
(Scharf et al., 2013; Sterlemann et al., 2008), mice were first tested on the EPM because performance in this test is highly sensitive to prior experiences (Crawley, 2008). The EPM consisted of two open (30 x 5 cm) and two closed arms (30 x 14.5 x 5 cm) elevated 42 cm off the ground. During the 5 min test, percent time on open arms, percent open arm entries, number of closed arm entries, and number of head dips were measured. Reduced percent time on/entries into open arms represent unconditioned anxiety which is independent of locomotion, whereas a reduction in closed arm entries signifies reduced locomotor activity (File, 2001). Arm entry was defined as two limbs passing the threshold of an arm.

**Sucrose Preference Test.** Depression-related behavior was assessed with the sucrose preference test (SPT). Reduced sucrose preference is widely considered a measure of anhedonia-related behavior (McCormick & Green, 2013). For SPT, mice at PND 66 were given 24-hr access to two bottles: one contained tap water and the other contained 1% sucrose. This concentration was previously shown to produce a robust sucrose preference (~85%) in BALB/cJ mice (Lewis et al., 2005). Bottle positions were switched after 12 h to control for side bias. Sucrose preference was quantified as: mass of sucrose solution intake (g)/total fluid intake (g)*100.

**Forced Swim Test.** Depression-related behavior was also measured in the forced swim test (FST) under dim red lights (~30-50 lux) during the active period (13:00 – 15:00 h). FST behavior was assessed 3 days after the SPT, on PND 69, to minimize the time that elapsed since the conclusion of CVSS. Previous studies have found little difference in performance when behavioral tests were administered with inter-test intervals of either 1-
2 days or 1 week (Paylor, Spencer, Yuva-Paylor, & Pieke-Dahl, 2006). The FST was conducted by placing mice into an inescapable glass beaker (18.5 wide x 24.5 cm high) filled with 25-27°C water for 6 min. The time spent immobile (s), defined as floating in an upright position and making only those movements necessary to keep its head above water, was measured during the last 4 min of the test period (Porsolt, Bertin, & Jalfre, 1977).

3.2.5. Statistics

Data were analyzed using two-way ANOVAs (sex x stress) or repeated measures ANOVA (time of day x sex x stress condition) where appropriate. Tukey’s HSD tests were used for post-hoc analyses and p-values < 0.05 were considered significant. Distributions of all variables were checked for normality. Outliers were detected using a Grubb’s outlier test (Grubbs, 1969) and excluded from analyses (sucrose preference test: 3/31 data points excluded; fCORT concentration: 1/64 data points excluded). Pearson’s correlation analysis was used to evaluate the relationship between fCORT concentrations and anxiety/depression-related behavior for males and females and females separately. Figures depict untransformed values. All analyses were performed and figures made using R v3.3.2.

3.3. Results

3.3.1. Fecal Corticoids

Analyses of peak and trough fCORT concentrations were performed separately for males and females due to a significant time x sex x stress condition interaction (F1,27=
10.09, p < 0.01). In males, fCORT concentrations were higher at the diurnal peak than trough (F1,14 = 41.07, p < 0.001). fCORT concentrations were significantly higher in CON males than CVSS males (Fig. 3-1A; F1,14 = 6.81, p < 0.05). In females, peak fCORT concentrations in CVSS mice were significantly higher than CON mice, but no difference was observed in trough fCORT concentrations (Fig. 3-1B; F1,13 = 10.61, p < 0.01). During the fCORT peak, male and female CVSS mice produced less fecal mass than CON mice (F1,23 = 4.81, p < 0.05; Mean ± S.E.M. for each condition: CVSS = 0.49±0.02, CON = 0.57±0.03 g dry weight). No other significant effects were observed for fecal mass produced.

3.3.2. Elevated Plus-Maze

Relative to CON mice, male and female CVSS mice exhibited a decrease in percent time spent on the open arms (Fig. 3-1C; F1,28 = 7.29, p < 0.05), decreased percent open arm entries (Fig. 3-1D; F1,28 = 19.31, p < 0.001), and fewer head dips (F1,28 = 27.37, p < 0.001; Mean ± S.E.M. for each condition: CON = 11.07±0.93, CVSS = 4.56±0.82). CVSS mice also exhibited decreased locomotion relative to CON mice, as indicated by a reduction in closed arm entries (Fig. 3-1E; F1,28 = 21.03, p < 0.001). There were no main effects of sex or interaction effects for percent time on open arms, percent open arm entries, closed arm entries, or head dips.
3.3.3. *Sucrose Preference Test*

CVSS females exhibited decreased sucrose preference in the SPT relative to CON females; this was not observed in males (Fig. 3-1F; $F_{1,24} = 9.11, p < 0.01$). There were no other significant effects for sucrose preference.

3.3.4. *Forced Swim Test*

In the FST, there was no effect of CVSS on depression-related behavior, but females exhibited increased immobility compared to males ($F_{1,28} = 9.79, p < 0.001$; Mean ± S.E.M. for each sex: Female = 148.11±13.50, Male = 88.42±13.50). There were no other significant effects on FST behavior.
Figure 3-2. Exposure to adolescent CVSS alters HPA function and affective-related behavior in adult mice. (A) Fecal corticoid metabolite (fCORT) concentrations were lower in CVSS males than CON males. (B) Diurnal peak fCORT concentrations were lower in CVSS female than CON females. (Note differences in Y axis scales between males and females). (C) In the EPM, CVSS mice spent less time on the open arms, (D) made fewer open arm entries, and (E) fewer closed arm entries than CON mice. In the SPT, CVSS females exhibited reduced sucrose preference relative to CON females (F). Bars represent mean ± SEM. ***p < 0.001, **p < 0.01, *p < 0.05.
3.3.5. Correlations

Correlation analysis revealed significant relationships between peak fCORT concentrations and behavior in the EPM and SPT (Table 2). Peak fCORT concentrations were positively correlated with closed arm entries on the EPM for both males ($R^2 = 0.38$, $p < 0.05$) and females ($R^2 = 0.28$, $p < 0.05$). Females also exhibited a significant positive correlation between peak fCORT concentrations and sucrose preference ($R^2 = 0.47$, $p < 0.05$). There were no other significant correlations between fCORT concentrations and anxiety- or depression-related behaviors.
Table 3-3. Correlations between fCORT concentrations and affect-related behaviors.

<table>
<thead>
<tr>
<th>Diurnal Peak fCORT vs.</th>
<th>Females</th>
<th>Males</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPM: Open Arm Time (%)</td>
<td>$r$</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>$R^2$</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>$p$-value</td>
<td>0.37</td>
</tr>
<tr>
<td>EPM: Open Arm Entries (%)</td>
<td>$r$</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>$R^2$</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>$p$-value</td>
<td>0.13</td>
</tr>
<tr>
<td>EPM: Head Dips</td>
<td>$r$</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>$R^2$</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>$p$-value</td>
<td>0.12</td>
</tr>
<tr>
<td>EPM: Closed Arm Entries</td>
<td>$r$</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td>$R^2$</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>$p$-value</td>
<td>0.04*</td>
</tr>
<tr>
<td>SPT: Sucrose Preference (%)</td>
<td>$r$</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td>$R^2$</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td>$p$-value</td>
<td>0.01*</td>
</tr>
<tr>
<td>FST: Time Spent Immobile (sec)</td>
<td>$r$</td>
<td>-0.20</td>
</tr>
<tr>
<td></td>
<td>$R^2$</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>$p$-value</td>
<td>0.46</td>
</tr>
</tbody>
</table>

Diurnal Trough fCORT vs.

| EPM: Open Arm Time (%) | $r$     | -0.08 | -0.24|
|                        | $R^2$   | 0.007 | 0.06 |
|                        | $p$-value | 0.77  | 0.36 |
| EPM: Open Arm Entries (%) | $r$     | -0.29 | 0.34 |
|                        | $R^2$   | 0.08  | 0.12 |
|                        | $p$-value | 0.27  | 0.20 |
| EPM: Head Dips        | $r$     | -0.17 | 0.04 |
|                        | $R^2$   | 0.03  | 0.002|
|                        | $p$-value | 0.53  | 0.86 |
| EPM: Closed Arm Entries | $r$     | -0.004 | 0.44|
|                        | $R^2$   | 0.000 | 0.19 |
|                        | $p$-value | 0.99  | 0.09 |
| SPT: Sucrose Preference (%) | $r$     | 0.003 | -0.08|
|                        | $R^2$   | 0.000 | 0.007|
|                        | $p$-value | 0.99  | 0.76 |
| FST: Time Spent Immobile (sec) | $r$     | -0.22 | -0.29|
|                        | $R^2$   | 0.05  | 0.09 |
|                        | $p$-value | 0.41  | 0.26 |

Pearson correlation coefficients and regression $R^2$ statistic comparing fCORT concentrations at the diurnal peak and trough to behavior in the EPM, SPT, and FST. Correlation coefficients were calculated for females and males separately. $n = 13-16$ pairs in each analysis. * indicates $p<0.05$. 
3.4. Discussion

Overall, there are several advantages to the adolescent CVSS model of stress vulnerability. CVSS induces sex-specific effects on anxiety- and depression-related behaviors in inbred adult mice, which allows for future studies to test mechanisms of susceptibility in both sexes. CVSS includes multiple adolescent social stressors (i.e. isolation and novel social partners) which target individual differences in behavioral and HPA responses to different kinds of social stressors (Caruso et al., 2014; Scharf et al., 2013; Schmidt, Trümbach, et al., 2010). Finally, CVSS successfully induced adult stress effects in an inbred mouse, whereas most prior studies have been conducted with outbred rats and mice, where genetic variability can influence susceptibility to adolescent stress (Scharf et al., 2013; Schmidt, Scharf, Liebl, et al., 2010; Schmidt, Trümbach, et al., 2010). We are unaware of any other study that has compared sex-specific impacts of chronic adolescent social stressors on adult behavior and physiology in inbred mice (i.e., in the absence of genetic variability). CVSS provides a useful model for future comparisons of strain- and sex-specific effects of stress on anxiety- and depression-related behaviors to elucidate genetic and environmental factors underlying stress-induced disorders.

The current study extends prior findings by showing that combined exposure to social isolation and novelty during adolescent development has some similar effects on adult male and female anxiety-related behavior in inbred BALB/cJ mice. Mice that experienced adolescent chronic variable social stress (CVSS) displayed altered affective-related behaviors during adulthood. When tested in the EPM, both male and female adult
mice exposed to CVSS exhibited a 35-40% reduction in open arm time and entries relative to CON mice. CVSS mice also exhibited reduced locomotor activity on the EPM which was recently acknowledged to be an important component of rodent anxiety-related phenotypes (Thompson, Grabowski-Boase, & Tarantino, 2015). C57BL/6J, BALB/cJ, and DBA/2J mice treated with prototypical anxiolytics exhibited increased locomotor activity in several anxiety-related behavior tests with no effect on standard anxiety-related behaviors (e.g., time in the center of an open field or open arm time in the EPM) (Thompson et al., 2015). Thus, CVSS-induced changes in locomotor activity in a novel environment are difficult to decouple from typical anxiety-related behaviors and reflect an anxiety-related state. The magnitude of the increase in anxiety-related behavior in the current study is comparable to prior studies that used different adolescent stress paradigms with outbred rodents (Table 1). Previous studies have shown that: (1) adolescent social isolation leads to ~25-30% increase in adult anxiety-related behavior in genetically diverse outbred male rodents (Lukkes et al., 2009; Ros-Simó & Valverde, 2012), (2) adolescent social instability increases adult anxiety-related behavior by ~25-60% in both male and female outbred animals (McCormick et al., 2008; Scharf et al., 2013; Schmidt, Scharf, Liebl, et al., 2010; Schmidt, Trümbach, et al., 2010), and (3) social defeat, and combined isolation, restraint, and social defeat (mixed modality stress) increase adult anxiety-related behavior by ~30-40% in adult male and female outbred rats (Bourke & Neigh, 2011; Vidal et al., 2007). Taken together, adult rodents exposed to stressful adolescent social experiences exhibit behavioral phenotypes indicative of heightened fear and anxiety.
Adolescent CVSS increased adult depression-related behavior in a sex- and test-dependent manner. We found a 20% reduction in sucrose preference in CVSS females which represents anhedonia-related behavior (McCormick & Green, 2013). Control males failed to exhibit a preference for 1% sucrose, whereas prior studies have indicated that BALB/cJ males show a strong preference at this concentration (Lewis et al., 2005). It is unclear why our results differ from this prior study, but because of the lack of preference in control males, we are unable to determine whether stress influenced male anhedonia-related behavior. There was no effect of adolescent social stress on FST behavior, but females exhibited more immobility than males. Similar results were previously reported in adolescent rats (Leussis & Andersen, 2008). The current SPT and FST results may suggest that female mice are more vulnerable to depression-related effects of adolescent social stress than males. Furthermore, enhanced susceptibility to isolation stress may have exacerbated behavioral changes in CVSS females contributing to reduced sucrose preference in mice necessarily isolated for the SPT. Sex differences have been observed for a number of different rodent models of depression-related behavior, but the direction of sex differences are highly dependent on the behavioral parameters that were investigated and the type/duration of stressors used (Dalla, Pitychoutis, Kokras, & Papadopoulou-Daifoti, 2012).

Few studies have assessed adult depression-related behavior following adolescent social stress. Similar to our findings, mixed modality adolescent stress reduced sucrose preference by ~25% and increased FST immobility by ~35% in adult outbred female rats, whereas males were unaffected (Bourke & Neigh, 2011). Social
instability also increased immobility in the tail-suspension test in outbred male mice, although females have not been assessed (Schmidt, Scharf, Liebl, et al., 2010). However, 20% of these mice were resilient to adolescent social stress and did not exhibit increased anxiety- or depression-related behavior (Scharf et al., 2013), and resiliency was associated with a heterogeneous genetic background (Schmidt, Trümbach, et al., 2010). These results accentuate the need to model adolescent social stress in both male and female inbred mice to control genetic variability. The current study suggests that adolescent social stress may lead to anxiety-related processes in both males and females, and to depression-related processes only in females.

Importantly, CVSS may have sensitized behavioral responses to subsequent social stressors. All mice were isolated for fCORT measures and then rehoused 24 hrs prior to EPM testing. Previous studies have found no effect of social isolation on anxiety-related behavior in adult BALB/cAnNCrl mice (Arndt et al., 2009), but we are unaware of any studies that have assessed the impact of a short 24-hr resocialization period on anxiety-related behavior. The magnitude of the CVSS-induced anxiety-related behavior in the current study may have been modulated by isolation and 24-hr resocialization prior to EPM. Previous studies have found that anxiety-related effects of adolescent stress are potentiated in adult mice re-exposed to a social stressor during adulthood (Jacobson-Pick, Audet, Nathoo, & Anisman, 2011). Future studies are necessary to evaluate this “two-hit” hypothesis whereby mice exposed to adolescent social stress show altered emotional responses when re-exposed to stressors later in life. Overall, the results of the current
study suggest that chronic variable social stressors lead to a complex anxiety-related behavioral profile in both male and female inbred mice (BALB/cJ).

Social experiences during adolescence shape adult HPA regulation (Bourke & Neigh, 2011; Caruso et al., 2014; Schmidt, Scharf, Liebl, et al., 2010). Prolonged GC exposure is one mechanism by which adolescent social stress may reprogram limbic brain development conferring vulnerability to anxiety and depression (McCormick et al., 2015b). CVSS was associated with reduced HPA activity in males and females, as indicated by lower fCORT concentrations at the end of adolescence. This effect was predominately due to reduced diurnal peak fCORT concentrations. CVSS mice also produced less fecal mass than CON mice during the diurnal peak, which could artificially elevate d fCORT concentrations (Cavigelli et al., 2005). Therefore, GC production during the diurnal peak may have been further diminished in CVSS mice than is evident from the fCORT measure. We also found dramatic sex differences in fCORT concentrations which were surprising because males typically excrete more fecal GC metabolites than females (Cavigelli et al., 2005; Touma et al., 2003). Results probably reflect sex differences in HPA responses to isolation which has no effect, or even reduces, GC production in males, but causes a substantial increase in females (Arndt et al., 2009; Kamakura, Kovalainen, Leppäluoto, Herzig, & Mäkelä, 2016; Palanza et al., 2001).

Given that adolescent social experiences prepare an organism for its future social environment, hypoactive adult HPA activity following social stress in adolescence may limit deleterious effects of prolonged GC exposure during potential stressful adult conditions. While reduced fCORT levels in CVSS males could indicate less adolescent
adversity, we do not believe this to be the case because increased anxiety-related behavior was also observed in these mice. Several studies have reported HPA hypoactivity in rats and mice exposed to adolescent social/non-social stress which are coupled with increased anxiety- and depression-related behavior (Jacobson-Pick et al., 2011; Ros-Simó & Valverde, 2012; Scharf et al., 2013). Moreover, adult male C57BL/6N mice with high basal CORT levels following chronic restraint stress exhibited reduced anxiety-like behavior and increased locomotion in the EPM and OFT relative to stressed mice with low basal CORT (Kim, Jung, Kim, Min, & Yoon, 2013). Significant correlations in peak fCORT concentrations with closed arm entries on the EPM and with sucrose preference further suggests that stress-induced HPA dysregulation is associated with individual differences in EPM and SPT behavior. Alternatively, previous experience with isolation for CVSS versus CON mice could have led to HPA habituation and reduced fCORT production following a week of isolation. Finally, adolescent social instability reduced basal corticosteroid-binding globulin levels resulting in increased free (i.e., bioactive) CORT among rats (McCormick et al., 2007). Whereas plasma CORT measurements reflect total CORT (i.e., free and bound CORT), fCORT measurements provide an index of only free, biologically-active, CORT levels (Cavigelli et al., 2005). As a result, we cannot rule out the possibility CVSS influenced the proportion of biologically active to inactive CORT levels. Further work is necessary to disentangle this relationship.
There are some limitations to consider when interpreting the current results. The use of a single inbred strain may limit the generalizability of our results, particularly because these effects were found in BALB/cJ mice that normally exhibit relatively high levels of anxiety/depression-related behavior (Belzung & Griebel, 2001; Jacobson & Cryan, 2007). The effects of adolescent stress may be potentiated in mice that display an anxious phenotype under basal conditions. For example, BALB/cOlaHSD and C57BL/6JOlaHsd adult males both exhibit depression-related behavior following exposure to chronic social defeat stress. However, these behavioral effects were only observed in BALB/cOlaHSD males following exposure to a less severe social defeat paradigm suggesting increased sensitivity to stress in this strain (Savignac et al., 2011). Furthermore, strain differences in anxiety/depression-related behavior have been associated with genetic polymorphisms known to modulate stress-susceptibility (Jacobson & Cryan, 2007). Future studies that incorporate multiple inbred and/or genetically modified mice will strengthen the translational value of our findings and provide valuable insight into the genetic and environmental factors that mediate CVSS-induced phenotypes.

A second limitation concerns the use of behavioral test batteries. Test batteries are an indispensable resource for characterizing behavioral phenotypes among a limited number of animals (Crawley, 2008). In the current study, behavioral tests were presented in the same order for all mice. It is therefore conceivable that prior experiences with novelty (i.e., EPM testing) could impact performance in subsequent tests. Few studies
have systematically evaluated test order effects within a test battery. In one study, few differences were found when performance was compared between 4 different behavioral test orders (McIlwain, Merriweather, Yuva-Paylor, & Paylor, 2001). In lieu of a more comprehensive understanding of test order effects, future studies which vary test orders within the experimental conditions could reduce the risk of generating results that are idiosyncratic to a particular test order.

3.4.2. Conclusion

The current study indicates that CVSS, a novel adolescent social stress protocol, induces sex-specific changes in depression-related behavior, but not HPA activity and anxiety-related behavior, in an inbred mouse strain. Results are consistent with findings in outbred rats and mice exposed to similar stress paradigms. The CVSS protocol may be beneficial for future studies to identify neurobiological mechanisms involved in sex-specific vulnerabilities to adolescent social stress.
3.5. References


Chapter 4. Adolescent chronic variable social stress influences exploratory behavior and nicotine responses in male, but not female, BALB/cJ mice.

4.1. Introduction

Tobacco use is the leading cause of preventable death in the United States. Approximately 480,000 Americans die each year as a result of smoking cigarettes (Center for Disease Control and Prevention, 2015). This public health problem does not exist in isolation; a strong bi-directional link has been established between affective disorders and nicotine use. Affective disorders, including depression and anxiety, are the second leading cause of global disease burden with an annual economic cost of $210 billion in the United States alone (Ferrari et al., 2013; Greenberg et al., 2015). Individuals who are diagnosed with an affective disorder are at higher risk of smoking compared to those without an affective disorder (Koenen et al., 2005; Swendsen et al., 2010). Conversely, higher risks of affective disorder diagnoses and greater symptom severity are reported in smokers compared to non-smokers (Grover et al., 2012; Jamal, Willem Van der Does, Cuijpers, & Penninx, 2012b; Richardson et al., 2012). However, it is unclear what factors promote the development of affective disorder and nicotine use comorbidity. Use of tobacco products could precipitate dysregulated mood (Boden, Fergusson, & Horwood, 2010). However, acute nicotine use has anxiolytic and antidepressant effects whereas nicotine withdrawal has been associated with increased anxiety and depressed mood (Hatsukami, Hughes, & Pickens, 1985; Molas et al., 2016; Tizabi et al., 1999). Thus, smoking could serve as a form of self-medication to ameliorate anxiety and
depression symptoms (Markou, Kosten, & Koob, 1998). More research is needed to investigate factors that predispose individuals to the development of both affective disorders and nicotine use.

Adolescence is a developmental period that is associated with a number of vulnerabilities. For example, problem nicotine use almost always begins in adolescence when there is a concomitant rise in the incidence of affective disorders (Kessler et al., 2005; Substance Abuse and Mental Health Services Administration, 2011). Chronic stress may be an important mediator of both affective disorders and nicotine use. Adolescent stress exposure often precipitates anxiety and depression (Andersen & Teicher, 2008), and predicts the initiation, degree, and continuation of smoking (Finkelstein et al., 2006; Rao et al., 2009b). Prospective clinical studies have attributed these stress effects to elevated glucocorticoid (GC) hormones which are released following stress-induced activation of the hypothalamic-pituitary-adrenal axis (HPA) (Rao et al., 2009b). Aberrant HPA activity may be particularly detrimental in adolescence because GCs play an important role in developmental programming of brain regions mediating both affect-related behavior and reward processing (i.e., the mesolimbic dopamine system) (Nestler & Carlezon, 2006; Placzek et al., 2009; Pryce, 2008; Yuan et al., 2015). Thus, adolescent stress could predispose individuals to develop affective disorders and nicotine use by altering development of biological processes involved in both conditions.

Rodent models of social stress have proved useful to investigate neurobiological factors mediating stress-induced affective disorders. In rodents, adolescence is often
defined as the period between postnatal days (PND) 21–59 (Tirelli et al., 2003). We recently reported that inbred male and female BALB/cJ mice exposed to adolescent chronic variable social stress (CVSS; repeated cycles of individual housing and exposure to novel social partner) exhibit sex-specific increases in anxiety- and depression-like behavior (Caruso, Kamens, & Cavigelli, n.d.). Our findings are in agreement with other studies in which rats and mice exposed to repeated social instability stress (e.g., repeated exposure to novel social partners) exhibit increased anxiety- and depression-related behaviors in adolescence and adulthood (Mathews et al., 2008; McCormick et al., 2008; Schmidt, Scharf, Liebl, et al., 2010; Sterlemann et al., 2008). Adolescent stress exposure can also increase or decrease nicotine responses under certain conditions. For instance, there were no effects of social instability, social defeat, or chronic restraint stress on nicotine-induced locomotion or nicotine self-administration in male rats (Cruz et al., 2008; McCormick & Ibrahim, 2007; McCormick et al., 2004, 2005; Zou et al., 2014). However, exposure to social instability during mid-adolescence blunted locomotor sensitization to repeated nicotine injections in late-adolescent (PND 58) female rats (McCormick & Ibrahim, 2007), had no effect at a lower dose in adulthood (PND 69) ~3.5 weeks after stress exposure (McCormick et al., 2005), and augmented sensitization in adult (PND 80) females tested ~5 weeks after stress exposure (McCormick et al., 2004).

Long-lasting HPA abnormalities frequently accompany adolescent stress-induced alterations in anxiety/depression-related behavior and nicotine responses. For example, adolescent and adult rats and mice exposed to social instability stress exhibit elevated basal corticosterone (CORT) levels (McCormick et al., 2007; Schmidt, Scharf,
Liebl, et al., 2010; Sterlemann et al., 2008). Curiously, our lab and others have also reported reduced HPA activity in adult males following adolescent social stress which may represent a protective mechanism to limit the deleterious effects of prolonged GC exposure (Bourke & Neigh, 2011; Caruso et al., under review.; Scharf et al., 2013). Stress-induced alterations in the expression of corticotropin releasing hormone and vasopressin, which regulate anxiety, depression, HPA activity, and many pharmacological effects of nicotine have also been reported (Lutfy et al., 2012; McCormick et al., 2007; Schmidt, Scharf, Liebl, et al., 2010; Sterlemann et al., 2008). To date, few studies have simultaneously investigated the influence of adolescent social stress on anxiety- and depression-related behavior, HPA activity, and nicotine responses in animal models.

In the current study, we sought to replicate and extend prior findings (Caruso et al., under review) by systematically assessing the effects of adolescent CVSS on both adult anxiety/depression-related behavior and nicotine responses. The CVSS protocol was previously developed to investigate the sex-specific effects of adolescent stress in inbred mice (i.e., animals with limited genetic variability) (Caruso et al., under review). Here, a within subjects experimental design was used to assess the relationship between anxiety/depression-related behavior and nicotine responses. Based on previous findings, we hypothesized that exposure to adolescent CVSS would: 1) increase anxiety and depression-related behaviors in male and female mice, 2) decrease nicotine responses during late adolescence or increase responses during adulthood in female mice, and 3)
increase the association between anxiety/depression-related behaviors and nicotine responses within individuals.

4.2. Materials and Methods

4.2.1. Animals

Male and female BALB/cJ mice (The Jackson Laboratory, Bar Harbor, ME) were bred at Pennsylvania State University. A total of 117 mice (51 females and 66 males) from 18 litters were used in the current study. Pups remained with the dam until weaning on PND 21, then were housed with 3-4 same-sex cagemates. Mice were housed in polycarbonate cages (28 cm x 17 cm x 12 cm) with corn-cob bedding in a temperature-controlled vivarium. Mice were maintained on a reverse 12:12 h light:dark schedule (lights on 13:00 h) with ad libitum food and water. All procedures were approved by the Pennsylvania State University IACUC committee. Mice were randomly assigned to either CVSS or control (CON) conditions (see section “Chronic variable social stress protocol”). Littermates were evenly distributed between groups in order to avoid bias due to litter effects.

4.2.2. Experimental design

Experiment 1 (Figure 4-1) was designed to determine whether adolescent CVSS would induce sex-specific changes in adolescent nicotine responses and affect-related behaviors in adulthood ($n = 11-15$/sex/condition). Specifically, we assessed nicotine-induced hypothermia and locomotion during late adolescence (PND 56-59) and voluntary oral nicotine consumption in adulthood (PND 116-135). Adult anxiety- and depression-
related behavior were tested in the EPM (PND 62-65) and SI test (PND 140-144), respectively.

Experiment 2 (Figure 4-1) was designed to determine whether adolescent CVSS would induce sex-specific changes in adult nicotine responses and adult affect-related behaviors ($n = 9-12$/sex/stress condition). In this experiment, acute nicotine responses were assessed in adulthood because prior studies reported that adolescent social stress can either reduce or augment locomotor sensitization to nicotine when tested during late-adolescence or adulthood, respectively (McCormick & Ibrahim, 2007; McCormick et al., 2004). Acute nicotine-induced hypothermia and corticosterone (CORT) production (PND 65-70) and voluntary oral nicotine consumption (PND 73-92) were measured during adulthood. As in experiment 1, adult anxiety- and depression-related behaviors were assessed in the EPM (PND 61-63) and SI test (PND 95-97).

Figure 4-1. Diagram illustrating experimental timelines. PND = Postnatal day; CVSS = Chronic Variable Social Stress
housing for 3 days followed by re-socialization with 1-2 unfamiliar same-sex cagemates (i.e., social reorganization) for 4 days. The social re-organization schedule was designed
such that the likelihood of a repeated encounter of the same mouse during the course of CVSS was minimized. CON mice remained housed with their original same-sex cagemates throughout the experiment. In order to limit differences in handling and husbandry between conditions, all CON mice were transferred to clean cages with their cagemates on days when CVSS mice were placed into individual-housing or social reorganization. On PND 59, CVSS mice were re-housed with their original cage-mates from weaning where they remained for the duration of the study unless otherwise specified (see below).

4.2.4. Behavioral Testing

With the exception of voluntary oral nicotine consumption testing, all behavioral testing was performed in a room that was separate from the colony room. On the morning of testing, mouse tails were marked with Sharpie® marker for easy identification and to limit handling stress prior to testing. Mice were transported to the behavior room at least 1 hr prior to testing to habituate them to the testing environment.

Acute nicotine responses. The acute effects of nicotine on locomotor activity, body temperature, and HPA activity were examined using a modified test battery (Marks, Romm, Bealer, & Collins, 1985; Marks, Stitzel, & Collins, 1989). Locomotor activity was examined under reduced anxiogenic conditions by measuring total distance traveled in a symmetrical Y-maze consisting of 3 red covered plexiglass arms (30 L x 8 W x 10 H; cm). Testing was performed in a brightly lit room (~600 lux), but light intensity was low inside the Y-maze (~30 lux). Trials were recorded by an overhead camera and analyzed
using an automated video-tracking system (Anymaze v.4.60, Stoelting, Wood Dale, IL). Body temperature was measured using a TH-5 Thermalert Monitoring Thermometer and a RET-3 mouse rectal probe (Physitemp Instruments Inc., Clifton, NJ, USA) lubricated with peanut oil. Nicotine-induced plasma CORT levels were measured 30 and 90 minutes following saline/nicotine injections in experiment 2. Baseline CORT levels were measured 3 days following the last nicotine/saline injection. All blood samples were obtained from 14:00-16:00h.

Nicotine effects on locomotion, body temperature, and HPA activity were measured using a within-subjects design (Kamens et al., 2015; Marks et al., 1989). Specifically, every animal in each experiment received i.p. injections of both saline and nicotine (Experiment 1 - 0.5 mg/kg; Experiment 2 - 0.5 or 1 mg/kg; doses presented as freebase nicotine). In both experiments, nicotine and saline injections were counter-balanced according to a Latin square design and testing sessions occurred 48 hours apart. In Experiment 1, a subset of mice (N= 11-12/sex/stress condition) were tested for acute nicotine responses. Immediately following injection, each mouse was placed into a Y-maze for 10 min. Locomotion was analyzed by measuring total distance traveled in the first 5 min of the test because near-maximal effects on Y-maze locomotion are observed 5 min after nicotine injection (Marks et al., 1985). Following locomotor activity testing, mice were returned to holding cages until rectal body temperature was measured 15 min after nicotine injection. At 30 and 90 min after injection mice were briefly restrained in a broom-style restrainer and blood was collected from the tail vein. A final blood sample for baseline CORT was collected 3 days after the last saline/nicotine injection. Mice were
transported in holding cages to a separate room for all blood collections. All samples were collected within 3 minutes of initial cage disruption.

Nicotine doses and testing times were based on published methods (Kamens et al., 2015; Marks et al., 1985, 1989). Two primary dependent variables were used to assess acute nicotine responses: nicotine-induced change in locomotor activity (cm) and change in body temperature (°C). Both variables were calculated as a within subject change score by subtracting the saline response from the nicotine response. Thus, positive locomotion values represent greater nicotine-stimulated activity and negative temperature values represent nicotine-induced hypothermia. In Experiment 2, data for adult nicotine-induced locomotor activity was excluded due to technical problems. Three primary dependent variables were used to assess HPA activity: plasma CORT levels at 30 min post-injection, plasma CORT levels at 90 min post-injection, and baseline plasma CORT levels.

**Two-bottle choice nicotine consumption.** Nicotine intake was measured in a standard 2-bottle free choice paradigm (Butt, King, Hutton, Collins, & Stitzel, 2005; Wilking, Hesterberg, Crouch, Homanics, & Stitzel, 2010). Animals were singly housed in standard mouse cages for testing. Mice were provided access to two 25 ml graduated cylinders fitted with drinking spouts filled with water for the first two days to acclimate them to the test environment. At the start of nicotine testing, one tube of water was replaced with a tube containing 25 mg/ml nicotine. Nicotine drinking solutions were made of free-base nicotine (Sigma Aldrich, St. Louis, MO) diluted in tap water (Matta et al., 2007). The volume of fluid in the tubes was recorded at approximately 14:00 h every day. The
left/right location of the nicotine and water-containing bottles was switched every other day to control for side bias. Nicotine was presented in concentrations (25, 50, 100 and 200 μg/ml) that increased every 4 days. Mice were weighed every 4th day when a new concentration of nicotine was presented and consumption data were adjusted for body weight. Consumption data were adjusted for evaporation/leakage by measuring fluid loss from 4 empty control cages that were handled the same as the experimental cages. Three primary dependent variables were obtained: nicotine consumption (mg/kg), nicotine preference (ml of nicotine/total ml of fluid), and total fluid consumed (ml). These dependent variables were derived from the average of days 2 and 4 of each nicotine concentration (i.e., the second full day after the bottle side or drug concentration was changed) (Phillips, Crabbe, Metten, & Belknap, 1994).

**Elevated plus-Maze.** Adult anxiety-related behavior was measured on the EPM as described previously (Caruso et al., *under review*). The 5 min test was conducted under dim red lights (~30 lux) between 9:00-12:00 h. The EPM was made from black Plexiglas and consisted of two open (30 L x 5 W; cm) and two closed arms (30 L x 14.5 H x 5 W; cm) elevated 42 cm off the ground. The maze was cleaned with 30% EtOH at the end of each trial. The primary dependent variables were percent open arm time, percent open arm entries, and number of closed arm entries. Reductions in percent time on and entries into the open arms represent unconditioned anxiety which is independent of locomotion (File, 2001; Rodgers & Dalvi, 1997). A reduction in closed arm entries signifies reduced locomotor activity which may also be indicative of an anxiety-related state (File, 2001; Rodgers & Dalvi, 1997; Thompson et al., 2015). Trials were recorded by an overhead
camera and behavior was analyzed using an automated video-tracking system. Arm entry was defined as 85% of the body passing the threshold of an arm. Percent open arm time was calculated as [Time on the open arms / (Time on open arms + Time on closed arms)] x 100. Percent open arm entries was calculated as [Total open arm entries / (Total open arm entries + Total closed arm entries)] x 100.

**Social interaction test.** The social interaction (SI) test was used to assess adult social approach/avoidance behavior. Testing was performed as previously described (Berton et al., 2006). Briefly, trials were recorded by an overhead camera and a video-tracking system was used to analyze social approach-avoidance behaviors and general locomotion in an open field arena (60 L x 60 L x 30 H; cm) made of white Plexiglas. Testing was performed under dim red lights (~30 lux) between 13:00-17:00 h. The test consisted of two consecutive 2.5 min trials during which mice were allowed to freely explore the arena. During the first trial (“no target”) an empty circular wire mesh cage (9 cm in diameter) was located at one end of the field. During the second trial (“target”) the empty cage was replaced with an identical cage containing a target mouse (an unfamiliar same-sex adult BALB/cJ mouse). At the end of the first trial, the test mouse was removed from the arena and placed back in the home cage for ~1 min. The primary dependent variables obtained to measure social approach/avoidance behavior were total distance travelled (cm), time spent in the interaction zone (5 cm corridor surrounding the cage) and time spent in the corner zones (8 x 8 cm) in the presence of the empty cage (“no target”) or a novel conspecific (“target”). Previous studies indicate that adult male C57BL/6J mice exposed to chronic social stress exhibit a profound and long-lasting reduction in time
spent in proximity to an unfamiliar social target which is interpreted as increased depression-related behavior (Berton et al., 2006).

4.2.5. **Radioimmunoassay**

Blood samples (20-50 μl) were obtained by tail cut, collected into heparinized capillary tubes (RAM Scientific, Yonkers, NY), and stored on ice. Samples were centrifuged at 4°C, plasma was collected and stored at -80°C until assayed. Plasma CORT levels were measured in duplicate using a commercially available [I^{125}] radioimmunoassay kit (MP Biomedicals, Solon, OH) according to the manufacturer’s instructions. Intra- and inter-assay coefficients of variation for low and high controls were 7.6 and 12.7, respectively. Additionally, area under the curve with respect to increase (AUC) (Pruessner, Kirschbaum, Meinlschmid, & Hellhammer, 2003) was calculated from the baseline, 30 min, and 90 min CORT levels to create an integrated measure of CORT response to each injection. CORT AUC was used for correlation analyses between EPM behavior, social interaction test behavior, and additional nicotine responses.

4.2.6. **Statistical analyses**

All statistical analyses were performed in R (v3.3.2). Dependent variables were analyzed using an analysis of variance (ANOVA) or a mixed factorial ANOVA with sex (male and female), stress condition (CVSS and CON), nicotine dose (Saline, 0.5 mg/kg, and 1.0 mg/kg), nicotine concentration (25 μg/ml, 50 μg/ml, 100 μg/ml, and 200 μg/ml), or time since injection (30 min and 90 min) as possible independent variables. Dependent
variables were checked for normality and outliers removed (± 1.5 * interquartile range) or
data was log transformed, where appropriate, to meet requirements for parametric
statistical analyses. Continuous litter mean values were calculated for each dependent
variable and included as covariates in all statistical models to control for litter effects.
Data in Experiments 1 and 2 were combined for analysis of body weight change, EPM
behaviors, and social interaction test behaviors with experimental cohort included as
factors in the statistical model unless there was a significant difference between
experimental cohorts. Nicotine responses were analyzed separately for each experiment
because previous studies have shown that the acute effects of nicotine differ considerably
in adolescent and adult rodents and adolescent nicotine exposure can alter subsequent
adult nicotine responses (Brielmaier, McDonald, & Smith, 2007; Cao et al., 2010; Nesil,
Kanit, Collins, & Pogun, 2011; Wilking et al., 2012). Whenever a significant main effect
or interaction was identified post-hoc analyses were performed using Tukey’s HSD. α <
0.05 was considered significant for all statistical analyses including post-hoc
comparisons. Partial correlations, controlling for litter, were calculated separately for
males and females in each experiment to test the relationship between
anxiety/depression-related behaviors and nicotine responses within individuals. For
clarity, figures depict estimated marginal means calculated from untransformed data.
4.3. Results

4.3.1. Locomotion and body temperature

Exposure to adolescent social stress affected nicotine-induced change in locomotor activity in late adolescence (Figure 4-2A). Late-adolescent CVSS males exhibited significantly greater nicotine-induced change in locomotor activity than CON males, but no difference was observed between CVSS and CON females (Figure 4-2A; Sex x stress condition interaction: $F_{1,40}= 6.3, p < 0.05$). There were no other main effects for nicotine-induced change in locomotor activity.

Exposure to adolescent social stress did not affect nicotine-induced hypothermia (Figure 4-2B-D). There were no effects of sex or stress condition on reduction in body temperature following 0.5 mg/kg nicotine injection in late adolescent mice (Figure 4-2B). During adulthood, there was a dose-dependent reduction in body temperature following nicotine 0.5 mg/kg and 1.0 mg/kg nicotine injections (Figure 4-2C-D; Main effect of nicotine dose: $F_{1,30}= 7.4, p < 0.01$). There were no other main effects or interactions on nicotine-induced hypothermia.
Figure 4-2. Adolescent social stress altered nicotine-induced changes in locomotor activity in late-adolescent male mice. CVSS did not influence nicotine induced change in body temperature for males or females during late-adolescence or adulthood. Data (mean ± SEM) represent (A) nicotine-induced locomotor activity in late-adolescent mice and nicotine-induced hypothermia in (B) late-adolescent mice and adult (C) male and (D) female mice. Bars represent nicotine response minus saline response. Thus, higher positive locomotion values indicate greater locomotor activity and more negative temperature values represent greater nicotine-induced hypothermia. *p < 0.05 depicting a significant sex x stress condition interaction on nicotine-induced change in locomotor activity. N = 11-12/Sex/Stress condition of late-adolescent mice and N = 9-12/Sex/Stress condition of adult mice.
4.3.2. Glucocorticoid production

Exposure to adolescent social stress affected adult GC production at baseline and in response to nicotine (Figure 4-3A). Adult CVSS mice had lower baseline CORT levels than CON mice (Figure 4-3A; Main effect of stress condition: F\(_{1,34}= 5.0, p < 0.05\)). There was no main effect of sex or interaction for baseline CORT levels.

Exposure to adolescent social stress increased nicotine-induced CORT production in a sex- and dose-dependent manner (Figure 4-3B-G). Analyses were performed separately at each dose for males and females due to a significant main effect of nicotine dose (F\(_{2,183}= 5.7, p < 0.01\)) and a significant time x sex x stress condition interaction effect (F\(_{1,183}= 4.48, p < 0.05\)). In males, plasma CORT levels were consistently higher at 30 min, relative to 90 min, for all doses (Figure 4-3B-D; Main effect of time: saline - F\(_{1,17}= 19.4, p < 0.001\); 0.5 mg/kg nicotine - F\(_{1,17}= 96.7, p < 0.001\) - 1.0 mg/kg nicotine: F\(_{1,16}= 80.8, p < 0.001\)). There was no effect of stress condition on plasma CORT levels following saline or 0.5 mg/kg nicotine. However, CVSS males had significantly higher plasma CORT levels than CON males 30 min following 1.0 mg/kg nicotine, with no difference 90 min following injection (Figure 4-3F; Time x stress condition interaction: F\(_{1,16}= 6.4, p < 0.05\)). In females, plasma CORT levels were consistently higher at 30 min, relative to 90 min, for all doses (Figure 4-3E-G; Main effect of time: saline - F\(_{1,20}= 31.9, p < 0.001\); 0.5 mg/kg nicotine - F\(_{1,20}= 29.5, p < 0.001\); 1.0 mg/kg nicotine - F\(_{1,20}= 22.4, p < 0.001\)). There was no effect of stress condition on plasma CORT levels following saline/nicotine injections in females.
4.3.3. Two-bottle choice nicotine consumption

Exposure to adolescent social stress decreased adult nicotine consumption in male, but not female mice, during Experiment 1 (Figure 4-4A-B). Analyses of nicotine consumption in Experiment 1 were performed separately for males and females due to a significant nicotine concentration x sex interaction ($F_{3,141} = 2.7, p < 0.05$). In males, nicotine consumption followed an inverted U-shaped dose-response curve. Males
consumed more nicotine when the 100 μg/ml concentration was available compared to the 25 μg/ml concentration (Main effect of nicotine concentration: $F_{3,60} = 3.8, p < 0.05$; 25 μg/ml: 0.7 ± 0.1, 50 μg/ml: 1.0 ± 0.1, 100 μg/ml: 1.2 ± 0.1, 200 μg/ml: 1.0 ± 0.1). CVSS males consumed less nicotine than CON males when the 200 μg/ml concentration was available (Figure 4-4A: Nicotine concentration x stress condition interaction: $F_{3,60} = 4.0, p < 0.05$). Females consumed less nicotine when the 200 μg/ml concentration was available compared to the 50 and 100 μg/ml concentrations (Main effect of nicotine concentration: $F_{3,81} = 6.8, p < 0.001$; 25 μg/ml: 0.7 ± 0.1, 50 μg/ml: 0.9 ± 0.1, 100 μg/ml: 1.1 ± 0.1, 200 μg/ml: 0.3 ± 0.1). There was no effect of stress condition on female nicotine consumption (Figure 4-4B).

Exposure to adolescent social stress decreased adult nicotine preference during Experiment 1 (Figure 4-4C-D). Nicotine preference decreased when 200 μg/ml nicotine was available compared to 25 μg/ml nicotine (Main effect of nicotine concentration: $F_{3,141} = 17.1, p < 0.001$; 25 μg/ml: 0.24 ± 0.03, 50 μg/ml: 0.21 ± 0.03, 100 μg/ml: 0.15 ± 0.02, 200 μg/ml: 0.06 ± 0.01). CVSS mice preferred less nicotine than CON mice when 200 μg/ml nicotine was available (Fig.4C-D; Nicotine concentration x stress condition interaction: $F_{3,141} = 3.1, p < 0.05$). There was no effect of sex on nicotine preference.

Exposure to adolescent social stress altered total fluid consumption in males, but not females, during Experiment 1. Analyses of total fluid consumption during Experiment 1 were performed separately for males and females due to a significant nicotine concentration x sex interaction ($F_{3,141} = 3.3, p < 0.05$). Males consumed more total fluid when 200 μg/ml nicotine was available compared to 25 μg/ml nicotine (Main
effect of nicotine concentration: $F_{3,60}= 4.2, p < 0.01$; 25 $\mu g/ml$: 6.0 ± 0.1, 50 $\mu g/ml$: 6.4 ± 0.1, 100 $\mu g/ml$: 6.5 ± 0.2, 200 $\mu g/ml$: 6.8 ± 0.3). Male CVSS mice also consumed more total fluids than CON mice (Main effect of stress condition: $F_{1,19}= 5.2, p < 0.05$; 6.7 ± 0.2 vs. 6.1 ± 0.2, respectively). There were no effects of nicotine concentration or stress condition on female total fluid consumption.

Exposure to adolescent social stress did not affect adult nicotine consumption, during Experiment 2 (Figure 4-4E-F). Analyses of nicotine consumption were performed separately for males and females due to a significant sex x stress condition interaction ($F_{1,36}= 7.1, p < 0.05$). Males consumed less nicotine when the 25 $\mu g/ml$ concentration was available compared to all other concentrations (Main effect of nicotine concentration: $F_{3,51}= 7.6, p < 0.001$; 25 $\mu g/ml$: 1.7 ± 0.2; 50 $\mu g/ml$: 0.8 ± 0.1; 100 $\mu g/ml$: 0.7 ± 0.1, 200 $\mu g/ml$: 2.1 ± 0.3). There was no effect of stress condition on male nicotine consumption (Figure 4-4E). Females consumed more nicotine when the 200 $\mu g/ml$ concentration was available compared to all other concentrations (Main effect of nicotine concentration: $F_{3,60}= 11.5, p < 0.001$; 25 $\mu g/ml$: 1.6 ± 0.2; 50 $\mu g/ml$: 1.0 ± 0.1; 100 $\mu g/ml$: 1.7 ± 0.2, 200 $\mu g/ml$: 3.5 ± 0.3). There was no effect of stress condition on female nicotine consumption (Figure 4-4F).

Exposure to adolescent social stress did not affect adult nicotine preference, during experiment 2 (Figure 4-4G-H). Analyses of nicotine preference were performed separately for males and females due to a significant stress condition x sex interaction ($F_{1,36}= 7.3, p < 0.05$). Males preferred more nicotine when the 25 $\mu g/ml$ concentration was available compared to all other concentrations (Main effect of nicotine concentration: $F_{3,51}= 7.6, p < 0.001$; 25 $\mu g/ml$: 1.7 ± 0.2; 50 $\mu g/ml$: 0.8 ± 0.1; 100 $\mu g/ml$: 0.7 ± 0.1, 200 $\mu g/ml$: 2.1 ± 0.3). There was no effect of stress condition on female nicotine preference (Figure 4-4H).
concentration: $F_{3,51} = 7.6, p < 0.001$; 25 μg/ml: 0.27 ± 0.04; 50 μg/ml: 0.13 ± 0.03; 100 μg/ml: 0.08 ± 0.01; 200 μg/ml: 0.11 ± 0.02). There was no effect of stress condition on male nicotine preference (Figure 4-4G). Female mice preferred more nicotine when the 25 μg/ml concentration was available compared to 50 and 200 μg/ml nicotine (Main effect of nicotine concentration: $F_{3,60} = 11.5, p < 0.001$; 25 μg/ml: 0.28 ± 0.03; 50 μg/ml: 0.08 ± 0.02; 100 μg/ml: 0.17 ± 0.04, 200 μg/ml: 0.06 ± 0.02). There was no effect of stress condition on female nicotine preference (Figure 4-4F).

Exposure to adolescent social stress did not affect total fluid consumption during Experiment 2. Mice consumed more total fluid when the 200 μg/ml nicotine concentration was available compared to 50 μg/ml nicotine (Main effect of nicotine concentration: $F_{3,111} = 4.1, p < 0.01$; 25 μg/ml: 5.8 ± 0.1; 50 μg/ml: 5.6 ± 0.1; 100 μg/ml: 6.0 ± 0.1, 200 μg/ml: 6.2 ± 0.1). There were no effects of sex or stress condition on total fluid consumption in Experiment 2.
4.3.4. Elevated plus-Maze

Exposure to adolescent social stress decreased locomotor activity on the EPM in male, but not female mice (Figure 4-5C). There was no effect of sex or stress condition on percent time on the open arms of the EPM (Figure 4-5A, all p’s > .10), but females exhibited greater percent open arms entries than males (Main effect of sex: F_{1,108} = 4.3, p
< 0.05; 10.9 ± 1.1 vs. 7.7 ± 1.0, respectively). This effect was driven by a sex difference in CON mice that was not observed for CVSS mice (Figure 4-5B; Stress condition x sex interaction: F<sub>1,106</sub> = 3.9, p < 0.05). Finally, CVSS males entered the closed arms less frequently than CON males, but no difference was observed between CVSS and CON females (Figure 4-5C; Stress condition x sex interaction: F<sub>1,106</sub> = 7.0, p < 0.01).

Figure 4-5. Adolescent CVSS altered locomotor activity on the elevated plus-maze (EPM) in male, but not female mice. Data (mean ± SEM) represent the (A) percent time spent on the open arms, (B) percent entries into the open arms, and (C) number of closed arm entries on the EPM. * denotes a significant sex x stress condition interaction (p < 0.05) on percent open arm entries. ** denotes a significant sex x stress condition interaction (p < 0.01) on closed arm entries. N = 24-31/Sex/Stress condition when data from mice in Experiments 1 and 2 were combined.

### 4.3.5. Social interaction test

Exposure to adolescent social stress decreased locomotor activity and inspection of novelty in the SI test (Figure 4-6A-B). Distance traveled in the social interaction test was significantly different between mice in Experiment 1 and 2 (F<sub>1,84</sub> = 33.27, p < 0.001). Mice in Experiment 1 traveled larger distances than mice in Experiment 2 (1292.3 ± 35.3
vs. 989.7 ± 37.1, respectively). However, data from both experiments were combined for further analyses because there was no interaction of experimental cohort with trial, sex, or stress condition, and similar results were obtained when experimental cohorts were analyzed separately. Overall, mice spent more time in the interaction zone when the social target was present compared to when the social target was absent (Main effect of trial: \( F_{1,84} = 110.0, \ p < 0.001; 45.5 \pm 1.9 \) vs. \( 24.5 \pm 1.0 \), respectively). Females spent more time in the interaction zone than males (Main effect of sex: \( F_{1,85} = 6.6, \ p < 0.05; 38.1 \pm 1.5 \) vs. \( 31.9 \pm 1.7 \)). CVSS males spent less time in the interaction zone than CON males, but there was no effect of stress condition in females (Figure 4-6A; Stress condition x trial interaction: \( F_{1,85} = 4.9, \ p < 0.05 \)). There were no significant main effects or interactions for time in the corner zones (all \( p \)'s > .10). Overall, mice were more active when the social target was absent compared to when the social target was present (Main effect of trial: \( F_{1,75} = 38.7, \ p < 0.001; 1248.8 \pm 30.4 \) vs. \( 1033.2 \pm 28.3 \), respectively), females were also more active than males (Main effect of sex: \( F_{1,84} = 9.6, \ p < 0.01; 1215.2 \pm 31.10 \) vs. \( 1066.9 \pm 34.8 \), respectively), and CON mice were more active than CVSS mice (Main effect of stress condition: \( F_{1,84} = 4.87, \ p < 0.05; 1199.0 \pm 33.5 \) vs. \( 1083.1 \pm 32.4 \), respectively). The effect of stress condition on distance traveled was driven by reduced locomotor activity in CVSS males, relative to CON males, whereas no difference was observed between CVSS and CON females (Figure 4-6B; Sex x stress condition interaction: \( F_{1,84} = 4.61, \ p < 0.05 \)).
Correlation analyses were performed to investigate the relationship between individual differences in EPM behaviors, SI test behaviors, and nicotine responses. Separate analyses were performed for males and females within each experiment because of sex-specific effects of adolescent social stress. Analyses were restricted to those behaviors that were influenced by adolescent social stress including closed arm entries on the EPM, distance traveled in the SI test, nicotine-induced locomotor activity, nicotine consumption, nicotine preference when the 200 μg/ml concentration was available, and CORT responses to the 1 mg/kg nicotine injection (Figure 4-7). The relationships

Figure 4-6. Adolescent CVSS altered inspection of novelty and locomotor activity in the SI test in male, but not female mice. Data (mean ± SEM) represents (A) the time mice spent in the interaction zone and (B) total distance traveled during the SI test. * denotes a significant sex x stress condition interactions ($ p < 0.05$) for time spent in the interaction zone and total distance traveled. N= 20-27/Sex/Stress condition when data from mice in Experiments 1 and 2 were combined.

4.3.6. Correlation analyses

Correlation analyses were performed to investigate the relationship between individual differences in EPM behaviors, SI test behaviors, and nicotine responses.
between all EPM and SI test behaviors and nicotine responses are included in the supplemental materials (Supplemental Figures S1-4).

Individual differences in locomotor activity in the EPM and SI test were associated with variation in nicotine responses among males. Males that exhibited increased nicotine-induced change in locomotion during late adolescence were less active in the SI test (Figure 4-7A; \( r = -0.48, p < 0.001 \)) and made more closed arm entries in the EPM (Figure 4-7B; \( r = 0.44, p < 0.05 \)) during adulthood. There was no association between late adolescent nicotine-induced change in locomotion and distance traveled in the SI test or closed arm entries on the EPM for females (Supplementary Fig. S2). Adult males with higher CORT responses to the acute 1.0 mg/kg nicotine injection also consumed more nicotine (Figure 4-7C; \( r = 0.54, p < 0.05 \)) and preferred more nicotine when 200 µg/ml concentration was available (Figure 4-7D; \( r = 0.54, p < 0.05 \)). Furthermore, there were no significant associations between closed arm entries on the EPM or distance traveled in the SI test and consumption of the 200 µg/ml nicotine solution or preference for the 200 µg/ml nicotine solution (Supplementary Fig. S1-4).

Exploratory analyses revealed associations between nicotine responses during late-adolescence and adulthood which were not predicted \textit{a priori}. During Experiment 1, females that exhibited less nicotine-induced change in locomotor activity during late-adolescence consumed more of the 200 µg/ml nicotine solution as adults (Supplementary Fig. S2; \( r = -0.47, p < 0.05 \)). Further, adult females that exhibited less nicotine-induced hypothermia also preferred more 200 µg/ml nicotine (Supplementary Fig. S2, \( r = -0.43, p < 0.05 \)).
Figure 4-7. Correlations between nicotine responses, locomotor activity the EPM, and locomotor activity in the SI test for male mice. Nicotine-induced change in locomotor activity during late-adolescence was (A) negatively correlated with distance traveled in the SI test (N = 15) and (B) positively correlated with closed arm entries in the EPM during adulthood (N = 21). Plasma CORT responses to 1.0 mg/kg nicotine was positively correlated with (C) consumption of 200 μg/ml nicotine (N = 16) and (D) preference for 200 μg/ml nicotine (N = 16). The shaded regions signify 95% confidence intervals.
4.4. Discussion

4.4.1. Overview

The current study simultaneously evaluated the impact of adolescent chronic variable social stress (CVSS) on anxiety and depression-related behaviors and nicotine responses in inbred mice. The results presented here provide the most comprehensive assessment of adolescent stress effects on nicotine responses to-date. Further, this is the first study, to our knowledge, that assessed the relationship between stress-induced changes in anxiety- and depression-related behaviors, physiology, and nicotine responses within the same animals. Exposure to adolescent social stress led to altered nicotine responses and reduced locomotor activity in the EPM and SI test in male, but not female, BALB/cJ mice. There were no effects of stress on prototypical anxiety- and depression-related behaviors. These results were unexpected given our previous findings and may be related to methodological differences between studies (Caruso et al., under review). However, mice exposed adolescent social stress in our prior study did exhibit reduced locomotor activity in the EPM (Caruso et al., under review). In the current study, similar reductions in locomotor activity in the EPM and SI test partially replicate and extend prior findings and suggest that males exposed to adolescent social stress exhibit a general reduction in novelty exploration. In males, the locomotor-stimulating effects of nicotine were moderately associated with novelty-evoked locomotor activity, but there was little evidence that individual differences in anxiety- or depression-related behavior were associated with nicotine responses.
4.4.2. Adolescent social stress alters nicotine responses in males

The effect of adolescent social stress on acute nicotine responses were assessed in late adolescence and adulthood. Adolescent nicotine responses differ considerably from that of adults and adolescents are more susceptible to develop nicotine dependence (Brielmaier et al., 2007; Cao et al., 2010; Nesil et al., 2011; Substance Abuse and Mental Health Services Administration, 2011; Wilking et al., 2012). Prospective clinical studies suggest that chronic stress exposure contributes to human adolescent susceptibility as it predicts the initiation, degree, and continuation of smoking (Finkelstein et al., 2006; Rao et al., 2009). In the current study, adolescent social stress augmented nicotine-induced locomotor activity in late-adolescent males, whereas females were unaffected. These results indicate that adolescent social stress can have a sex-specific impact on behavioral and physiological responses to nicotine, but these effects vary depending on the nicotine response measured.

There is scant evidence that adolescent stress impacts nicotine responses in rodent models. Most studies have specifically investigated the impact of stress on nicotine’s locomotor effects and no previous studies have assessed stress effects on nicotine-induced hypothermia. The available literature suggests that females may be more susceptible than males. McCormick and colleagues found that adolescent social instability stress reduced locomotor sensitization to repeated nicotine (0.5 mg/kg) exposure when administered during adolescence while there was no effect on acute locomotor responses to the initial exposure (McCormick & Ibrahim, 2007). When nicotine (0.5 mg/kg) was administered in adulthood, the initial locomotor response and
locomotor sensitization were augmented in stressed female rats. These effects were specific to a higher nicotine dose as stress did not affect locomotor responses at a lower dose (0.25 mg/kg) (McCormick et al., 2004, 2005). Neither social instability stress nor chronic restraint stress influenced nicotine-induced locomotor activity among adolescent and adult male rats (Cruz et al., 2008; McCormick & Ibrahim, 2007; McCormick et al., 2004, 2005).

There are a number of factors that could contribute to the differences observed between our results and those of previous studies. For example, nicotine-induced locomotor responses may be influenced by the types of testing apparatus used across studies. We measured nicotine-induced locomotor activity in a symmetrical Y-maze whereas previous studies measured locomotor responses in open field arenas. We also measured locomotor activity for 5 mins, which corresponds to the time when near-maximal effects of nicotine are observed (Cao et al., 2010; Marks et al., 1985), whereas other studies have measured cumulative distance traveled over 30-60 min (Cruz et al., 2008; McCormick et al., 2004, 2005, 2008). As such, it is difficult to compare our results with those of previous studies. Finally, nicotine responses are highly dependent on dose and genetic background (Marks, 2013), which varies across studies. We assessed locomotor responses to 0.5 mg/kg nicotine in inbred BALB/cJ mice, whereas previous studies evaluated responses to 0.25-0.5 mg/kg nicotine in outbred rats. Future studies which utilize a more thorough dose-response curve and/or multiple inbred strains would provide valuable insight into the potential factors contributing to the variability in results across studies.
Several studies have reported elevated CORT levels following nicotine administration (Cao et al., 2010; Cruz et al., 2008; Donny et al., 2000; Lutfy et al., 2012). GCs modulate many of the pharmacological effects of acute and chronic and nicotine exposure, including locomotor activity, hypothermia, and development of tolerance (Caggiula et al., 1998; Pauly, Grun, & Collins, 1992; Pauly, Ullman, & Collins, 1988). Stress-induced GC production may also contribute to the reinforcing properties of nicotine (Pauly et al., 1992; Pomerleau & Pomerleau, 1991). Thus, nicotine’s ability to stimulate HPA activity influences risk for dependence. Furthermore, GCs program the development of HPA regulatory mechanisms during adolescence and chronic stress effects on human adolescent smoking have been attributed to elevated GC production (Pryce, 2008; Rao et al., 2009). Given previous reports long-lasting stress-induced changes in HPA activity (McCormick et al., 2007; Schmidt et al., 2010; Sterlemann et al., 2008), we hypothesized that adolescent social stress would increase adult GC responses to nicotine. In support of our hypothesis, adult CVSS males exhibited greater plasma CORT responses to high dose nicotine (1.0 mg/kg). It is unlikely that this effect was a result of general enhancement in HPA responses to stressful per se, because there was no effect of adolescent social stress on plasma CORT levels following injections of saline or a lower nicotine dose (0.5 mg/kg). As such, the effects of adolescent stress exposure may only become apparent with higher nicotine doses. Curiously, baseline plasma CORT levels were decreased by adolescent social stress. This effect was mainly driven by females and probably did not influence male HPA responses to nicotine. Enhanced HPA responses to nicotine may contribute to the associations between adolescent stress and smoking risk reported in prospective clinical studies (Finkelstein et al., 2006; Rao et al.,
Future studies are necessary to determine whether CVSS effects on late-adolescent locomotor responses to nicotine are mediated by increased HPA activity.

Voluntary oral nicotine consumption was measured during adulthood to assess long-lasting stress-induced behavioral changes. Adolescent social stress reduced voluntary nicotine consumption and preference in adult males, but not females, when the highest nicotine concentration (200 µg/ml) was available. Interestingly, these stress-effects only emerged after mice had been exposed to nicotine during late-adolescence (Experiment 1) as opposed to those for which initial exposure occurred in adulthood (Experiment 2). Although these results should be interpreted cautiously, they suggest that adolescent nicotine exposure during social stress may interact to shape subsequent nicotine responses. Brief nicotine exposure can induce long-lasting enhancement of excitatory inputs into the mesolimbic dopamine system of young animals (Mansvelder & McGehee, 2000). Furthermore, nicotine injections administered in adolescence, but not adulthood, increase sensitivity to nicotine’s reinforcing properties when animals are re-exposed later in adulthood (Adriani, Deroche-Gamonet, Le Moal, Laviola, & Piazza, 2006; Kota, Robinson, & Imad Damaj, 2009). Even a single exposure to nicotine during adolescence has been shown to alter adult nicotine responses (Belluzzi, Lee, Oliff, & Leslie, 2004; Brielmaier et al., 2007), highlighting the importance of age of initial exposure as a predictor of subsequent responses. The current results suggest that age at one’s initial exposure to nicotine may be an important mediator of adolescent stress effects on voluntary oral nicotine consumption during adulthood.
The only previous study, to our knowledge, which investigated adolescent stress effects on nicotine intake found no difference in acquisition of intravenous (IV) self-administration between socially defeated and control male rats (Zou et al., 2014). Comparisons of results between IV and oral self-administration experiments are complicated by a number differences between these models. For example, whether oral consumption results in central nicotine concentrations that are behaviorally relevant or reflects nicotine’s reinforcing properties is unclear (Collins, Pogun, Nesil, & Kanit, 2012; Robinson, Marks, & Collins, 1996). Sensitivity to nicotine’s aversive properties, including nicotine-induced seizures and its bitter taste, also modulate oral consumption (Robinson et al., 1996). If chronic adolescent stress effects are similar to those previously reported following acute stress (e.g., stress enhances nicotine’s reinforcing properties (Brielmaier, McDonald, & Smith, 2012)), CVSS males may consume less nicotine than CON males because the desired effect is acquired at lower doses. This interpretation would be consistent with the enhanced sensitivity to nicotine reinforcement observed when initial nicotine exposure occurred in adolescence (Adriani et al., 2006; Kota et al., 2009). Alternatively, we cannot rule out the possibility that CVSS altered sensitivity to the drug’s aversive properties.

Stress-induced alterations in the development of distinct neurobiological circuits associated with dopamine transmission and/or nicotinic acetylcholine receptor (nAChR) function could influence nicotine responses. Increased locomotor responses to nicotine observed in the absence of a stress effect on nicotine-induced hypothermia may reflect a change in mesolimbic dopamine transmission. The most well-known mediators of
nicotine-induced locomotor activity, hypothermia, voluntary oral nicotine consumption, and HPA activation are nAChRs containing α4 and β2 subunits distributed across distinct neural circuits (Collins et al., 2012; Marks, 2013; Matta, Fu, Valentine, & Sharp, 1998). Nicotine effects on locomotion may contribute to nicotine reinforcement and reflects, in part, activation of the mesolimbic dopamine system by α4β2-containing nAChR on dopaminergic neurons (McGranahan, Patzlaff, Grady, Heinemann, & Booker, 2011; Picciotto et al., 1998; Tritto et al., 2004). However, nicotine-induced hypothermia is not mediated by α4β2-containing nAChR regulation of dopamine transmission (McGranahan et al., 2011). While this is only one potential mechanism by which this effect may occur, the plausibility of this hypothesis is supported by the fact that dopamine systems (e.g., cortical and mesolimbic dopamine) and nAChR function actively develop during adolescence (Burke & Miczek, 2014; Yuan et al., 2015). Furthermore, adolescent social stress reduces cortical extracellular dopamine levels which could indirectly enhance nicotine-induced dopamine release in the nucleus accumbens (Niwa et al., 2013; Watt, Burke, Renner, & Forster, 2009).

4.4.3. Adolescent social stress alters exploratory behavior in adult males

Adolescent social stress reduced closed arm entries on the EPM, but, surprisingly, did not influence prototypical anxiety and depression-related behaviors. BALB/cJ mice are characterized by high baseline anxiety-related behavior relative to other inbred mouse strains (e.g., low-anxiety C57BL/6J mice) (Cavigelli, Michael, & Ragan, 2013). This anxious phenotype may have limited our ability to detect an anxiogenic effect of adolescent social stress. We previously reported that adult BALB/cJ mice exposed to
CVSS exhibited reduced open arm activity on the EPM in addition to fewer closed arm entries (Caruso et al., n.d.). Methodological differences between studies may explain these contrasting results. Mice in our previous study were isolated for 7 days, at the end of the CVSS protocol, and were rehoused 24 hr prior to EPM testing. Stress from isolation/re-socialization could have contributed the reduction in open arm activity that we previously observed. In contrast, stable reductions in closed arm entries observed in adult CVSS males partially replicate the results of our prior study (Caruso et al., *under review*). Closed arm entries in the EPM is a classically interpreted as general locomotor activity that is distinct from the prototypical anxiety-related behaviors (File, 2001). Notably, a recent study found that several prototypical anxiolytics consistently increased locomotor activity in C57BL/6J, BALB/cJ, and DBA/2J mice across 4 anxiety-related behavioral tests. No changes were observed for prototypical anxiety-behaviors (e.g., open arm time on the EPM) (Thompson et al., 2015). Therefore, reductions in locomotor activity are difficult to distinguish from prototypical anxiety-related behaviors and may reflect an anxiety-related state.

Reduced locomotor activity was also observed in CVSS males during the SI test 1-2 months following the conclusion of stress as indicated by a general reduction in locomotor activity. The stress-induced reduction in locomotor activity is similar to the reduction in closed arm entries in the EPM and suggests that stress effects persist well into adulthood. Reduced exploratory behavior in the novel SI test arena would explain the significant reduction in time CVSS males spent in the interaction zone regardless of whether or not the social target was present. Previous studies have found that exposure to
chronic social defeat during either adolescence or adulthood will reduce the amount of time that male C57BL/6J mice spend interacting with a novel social partner with no effect on behavior when the social target is absent or general locomotor activity (Berton et al., 2006; Iñiguez et al., 2014). Although strain differences exist, similar results have been observed in adult BALB/cOlaHSD mice (Savignac et al., 2011). Thus, CVSS-induced behavioral changes observed in the current study in the SI test do not represent a depression-related phenotype. The current results partially replicate and extend findings from our prior study (Caruso et al., under review). Overall, adult CVSS males appear to exhibit a persistent low-exploration phenotype, perhaps when faced with novel or anxiogenic situations.

4.4.4. Associations between exploratory behavior and nicotine responses

Analyses of the association between stress-induced changes in exploratory behavior and nicotine responses revealed that individual differences in late-adolescent nicotine-induced change in locomotor activity predicted adult exploratory behaviors in two unfamiliar test apparatus. Whereas females showed no association between nicotine responses and EPM or SI test behavior, males exhibiting greater nicotine-induced change in locomotor activity were also less active in the SI test and more active in the EPM. These results may indicate that a common underlying neurobiological mechanism influences both novelty-evoked locomotor activity and nicotine-induced change in locomotor activity among males.
There are several potential explanations for these associations. Prior studies have indicated that hypoactive nucleus accumbens dopamine transmission causes reduced locomotor activity in a novel environment such as the SI test arena (Ikemoto & Panksepp, 1999). The nAChR subunits known to modulate nicotine-induced nucleus accumbens dopamine release (e.g., α4, α6, and β3 (Grady et al., 2007)) also regulate novelty-evoked locomotor activity. For example, mice that harbor genetic mutations in the α4, α6, or β3 subunit genes exhibit increased locomotion or reduced habituation of locomotor activity in unfamiliar test arenas (Cui et al., 2003; Drenan et al., 2008; Labarca et al., 2001). This overlap is notable in light of recent studies that demonstrate developmental changes in nAChR function and nicotine’s ability to stimulate dopamine release in the ventral striatum (Yuan et al., 2015). Altered development of striatal nAChR function following adolescent stress may, therefore, contribute to correlated changes in locomotor activity in a novel environment and nicotine responses.

### 4.4.5. Conclusion

The current study has provided evidence that adolescent CVSS can have sex-specific impacts on the development of nicotine responses, with little effect on adult anxiety and depression-related behaviors in males. Alternatively, reduced baseline HPA activity in the absence of altered affect or nicotine-related behaviors may suggest that CVSS is not a potent stressor to females. The literature suggests that chronic stress and HPA hyperactivity may predispose individuals to develop both affective disorders and nicotine use because GCs program the development of brain regions mediating both emotion-related behavior and reward processing (Nestler & Carlezon, 2006; Placzek et
al., 2009; Pryce, 2008; Yuan et al., 2015). However, the current results suggest that stress-induced affective disorders and elevated risk for nicotine use may also have unique biological mediators as adolescent stress had no impact on prototypical anxiety or depression-related behaviors, but reduced nicotine intake and augmented HPA responses (i.e., increased sensitivity) to nicotine in male, but not female, BALB/cJ mice.
4.5. References


Chapter 5. Summary of Findings and General Discussion

In the final chapter, I will summarize the findings of the experiments presented and suggest future research that can expand on these findings. The aims of this dissertation were (1) to clarify factors that contribute to variability in the effects of adolescent social stress and (2) to develop a rodent model of adolescent social stress that capitalizes on the influence of variability in behavior and physiology among individuals. Our rodent model was then used to investigate the impact of adolescent social stress on affect-related behavior, hypothalamic-pituitary-adrenal axis (HPA) activity, and nicotine responses. Our results suggest that individual differences in exploratory behavior (i.e. temperament) modulate the impact of adolescent social experiences on adult HPA activity. We also found that adolescent social stress can influence adult affect-related behavior and nicotine responses. However, these findings were complex and several questions remain unanswered.

5.1. Summary of findings

To determine whether individual differences in temperament modulate the impact of adolescent social experiences, male Sprague-Dawley rats that exhibited high (neophilic) or low (neophobic) levels of exploratory behavior were housed with littermates, novel social partners, or individually during adolescence. We then assessed adult behavior and HPA activity in response to a social challenge. In Chapter 2, we demonstrated the neophilic rats respond differently to housing with familiar social partners, novel social partners, or individual housing during adolescence than do
neophobic rats. Specifically, neophobic rats exposed to novel social partners exhibited transient reductions in exploratory behavior during early adulthood and delayed recovery of HPA responses to a social challenge compared to neophilic rats housed under the same condition. Alternatively, neophilic rats that remained with littermates exhibited a transient reduction in exploratory behavior. The HPA response to an adult social challenge also recovered more quickly in neophilic rats, compared to neophobic rats, housed with novel social partners in adolescence. Results suggest that individual differences in exploratory behavior (temperament) may modulate the influence of adolescent experiences on adult behavioral and adrenocortical function.

In Chapter 3, we sought to develop a rodent model of adolescent social stress that might control for the influences of temperament and/or other sources of variability on anxiety and depression-related behavior and physiology. The cause of the individual differences in temperament described in Chapter 2 are unclear, but could be due to genetic variability among the outbred rodents commonly used for adolescent social stress studies. Additionally, few studies have investigated sex differences in responses to adolescent social stress effects on adult behavior and physiology. We sought to control for genetic variability by using an inbred mouse strain (BALB/cJ) and individual differences in temperament by repeatedly exposing mice to both novel social partners and social isolation. These social stressors are commonly employed in the literature and have also been considered sex-specific stressors. Namely, isolation is more stressful for female mice and novel unfamiliar social partners may be more stressful for male mice (Arndt et
We found that adolescent social stress increased anxiety-related behavior and reduced locomotor activity on the elevated plus-maze (EPM) for both males and females. Females also exhibited an increase in depression-related behavior in the sucrose preference test. Finally, both males and females exposed to adolescent social stress exhibited reduced adult HPA activity. We reasoned that this novel adolescent social stress protocol may be a valuable tool for future studies investigating the impact of disruptions to adolescent social development on adult behavior and physiology.

In Chapter 4, we investigated whether social stress-induced anxiety and depression-related behaviors were related to altered nicotine responses among inbred BALB/cJ mice by using a within-subjects experimental design. We exposed male and female BALB/cJ mice to adolescent social stress using similar methods as described in Chapter 3.

Surprisingly, the results of this study only partially replicated our previous findings (Chapter 3). We did not find any changes in anxiety-related behavior in the EPM, depression-related behavior in the social interaction (SI) test, or nicotine responses among females. In males, exposure to adolescent social stress reduced locomotor activity in the EPM and SI test, but did not influence prototypical anxiety and depression-related behaviors. On the other hand, males exposed to adolescent social stress displayed enhanced nicotine-induced locomotor activity during late-adolescence and nicotine-induced HPA activity during adulthood. These mice also voluntarily consumed less
nicotine during adulthood than controls. Individual differences in exploratory behavior in the EPM and SI test were correlated with nicotine-induced locomotor activity, but we found little evidence of a relationship between anxiety and depression-related behaviors and nicotine responses. Our results suggest that there may be unique biological mediators that underlie stress-induced affective disorders and elevated risk for nicotine use.

5.2. Limitations

In these studies, there are several limitations, two of which I will discuss further. The first limitation concerns findings from Chapter 3 that were not replicated in Chapter 4. In particular, the only effect of adolescent CVSS that was observed in female mice from Chapter 4 was a reduction in baseline plasma corticosterone (CORT) levels. In Chapter 3, we found reduced baseline levels of fecal corticoid metabolites (fCORT) among males and females.

These differences may be due to several factors including methodological differences between the two experiments. Plasma CORT measurements provide an index of total CORT (i.e. free and bound CORT) at the time the sample was obtained. The fCORT method provides an integrated measure of free CORT (i.e. not bound by corticosteroid binding globulin - i.e. biologically active), that was metabolized and excreted over a discrete period of time (Cavigelli et al., 2005). Moreover, adolescent social stress was found to reduce plasma corticosteroid-binding globulin levels (McCormick et al., 2007). As a result, we cannot rule out the possibility CVSS influenced the proportion of biologically active to inactive CORT levels in Chapter 3.
Social housing requirements associated with the two methods of CORT measurement should also be considered. In order to measure fCORT in Chapter 3, mice were necessarily isolated for 9 days following adolescent CVSS. Mice in Chapter 4 were not isolated before blood sampling for plasma CORT measurements. In rodents, there are profound sex differences in HPA responses to individual housing. Isolation has no effect, or even reduces, CORT production in males, but causes a substantial increase in females (Arndt et al., 2009; Kamakura et al., 2016; Palanza et al., 2001). We also found dramatic sex differences for fCORT concentrations in Chapter 3 which were surprising because males typically excrete more fCORT than females (Cavigelli et al., 2005; Touma, Sachser, Möstl, & Palme, 2003).

Another major difference in results was the behavioral effects of adolescent CVSS in Chapters 3 and 4. In Chapter 3, males and females exposed to adolescent CVSS exhibited a reduction in open arm activity and reduced closed arm entries on the EPM. In Chapter 4, adolescent CVSS had no effect on female open arm activity, but males displayed a reduction in closed arm entries. It is possible that adolescent CVSS sensitized mice to the anxiogenic effects of subsequent social stressors. A similar two-hit hypothesis has been proposed in which the effects of adolescent stress are only evident or exacerbated when animals are re-exposed to stress in adulthood (Jacobson-Pick, Audet, Nathoo, & Anisman, 2011). For example, females may have been sensitized to the anxiogenic effects of social isolation (Arndt et al., 2009; Palanza et al., 2001). Males, on the other hand, are typically unaffected by social isolation, but the stress of a brief 24 hour re-socialization period, during which fighting was usually observed (unpublished
observation), prior to EPM testing may have contributed to our results. Future studies are necessary to adequately test this hypothesis.

Although increased prototypical anxiety-related behaviors were not observed in Chapter 4, we found long-lasting reductions in exploratory behavior that partially replicate the results of chapter 3. Males exposed to adolescent CVSS exhibited reduced locomotor activity in the EPM and the SI test (when tested at least 1 month later). These results suggest that adolescent CVSS induces a long-lasting phenotype characterized by reduced exploration of a novel environment. Locomotor activity is also an important component of anxiety-related behavior. In a recent study, BALB/cJ, C57BL/6J, and DBA/2J mice treated with several different anxiolytic drugs exhibited increased locomotor activity in several anxiety-related behavior tests. However, no differences were found in prototypical measures of anxiety-related behavior (i.e., time on open arms of the EPM) (Thompson, Grabowski-Boase, & Tarantino, 2015).

The second limitation concerns the generalizability of our findings. In Chapter 2, we found that outbred (i.e., genetically variable) rats exhibit individual differences in exploratory behavior which modulate the impact of adolescent social experiences on adult behavior and physiology. However, a single inbred mouse strain was studied in Chapters 3 and 4. In these experiments, we used BALB/cJ mice which are characterized as a high-anxiety strain that is more susceptible to the effects of stress than are other strains such as the C57BL/6J strain (Belzung & Griebel, 2001; Jacobson & Cryan, 2007). Strain differences in stress-susceptibility could have a strong impact on CVSS effects on the behavioral and physiological outcomes studied in this dissertation. The translational
significance of these findings could be greatly improved by studying the effects of CVSS on different strains of inbred mice.

5.3. Future directions

Future research should continue to investigate the impact of adolescent social stress on affect-related behavior and nicotine responses. There are two lines of research that are particularly relevant:

5.3.1. Evaluating the mechanism underlying the impact of adolescent CVSS on altered nicotine responses

What are the biological mechanisms associated with adolescent CVSS effects on male nicotine responses observed in Chapter 4? There are many possible mechanisms that explain these findings which are probably not mutually exclusive. In this section, I will propose mechanisms that may underlie stress-induced enhancement in locomotor responses to nicotine presented in Chapter 4. Of the many potential mechanisms, the most parsimonious explanation could relate to CVSS-induced alterations in cortical dopamine (DA).

As discussed in Chapter 1, the medial prefrontal cortex (mPFC) DA system matures considerably during adolescence (Andersen & Gazzara, 1993; Andersen et al., 2000; Naneix et al., 2012; Tarazi et al., 1998; Teicher et al., 1993). This maturational process appears to be disrupted by adolescent social stress. Adolescent social defeat or social isolation reduced extracellular DA levels at baseline and blunted amphetamine-induced DA release in the mPFC (Burke et al., 2013, 2010; Niwa et al., 2013; Watt et al., 2009). Like amphetamine, albeit through a different mechanism, acute nicotine injections
stimulate DA release at VTA-mPFC terminals (Nisell, Nomikos, Hertel, Panagis, & Svensson, 1996). Therefore, adolescent CVSS may have similar effects on basal and nicotine-induced mPFC DA in BALB/cJ males.

The locomotor effects of psychostimulant drugs are modulated by mPFC DA signaling. Under normal conditions, DA inhibits mPFC glutamatergic projection neurons (Tseng & O’Donnell, 2007) effectively suppressing the activity of a major source of NAc glutamate afferents. Pharmacological inhibition of NAc glutamate receptors also reduces locomotor responses to psychostimulants including amphetamine and nicotine (Scofield et al., 2016). Therefore, adolescent stress-induced reductions in mPFC DA activity may disinhibit cortical-accumbens glutamate projections to enhance glutamate release in the NAc.

Reduced mPFC DA activity could explain the augmented locomotor responses to nicotine that we observed in Chapter 4. Even brief stimulation of cortical-accumbens glutamate transmission can induce synaptic plasticity and augment psychostimulant-induced NAc DA release (Scofield et al., 2016). Data suggest that, like amphetamine, nicotine-induced locomotor responses reflect enhanced DA release in NAc (Clarke, 1990). Acute nicotine injections increase extracellular glutamate levels which also parallel locomotor responses (Lenoir & Kiyatkin, 2013; Reid, Fox, Ho, & Berger, 2000). Similar to the stress-effects on amphetamine responses, adolescent social instability stress enhances nicotine-induced locomotion (McCormick et al., 2004). Furthermore, blunted amphetamine-induced DA release in the mPFC, following adolescent social defeat, was negatively correlated with amphetamine-induced locomotor activity (Burke et al., 2013).
Reductions in D2 autoreceptor activity have been proposed to mediate stress effects on mPFC DA activity and locomotor responses to psychostimulants (Burke & Miczek, 2014). Adolescent social stress repeatedly stimulates phasic DA release in the mPFC (Burke et al., 2013; Watt et al., 2014). Phasic stress-induced DA release would repeatedly stimulate synthesis regulating autoreceptors which are transiently upregulated in the adolescent mPFC (Andersen, Dumont, & Teicher, 1997). Activation of these D2 autoreceptors inhibits DA synthesis via inhibition of tyrosine hydroxylase activity (Lindgren et al., 2003). These effects may be permanent as tyrosine hydroxylase and mPFC DA levels are reduced in adult animals that were exposed to the D2 agonist quinpirole during adolescence (Naneix, Marchand, Pichon, Pape, & Coutureau, 2013; Watt et al., 2014). Finally, pharmacological inhibition of D2 receptors, via intra-mPFC infusions of amisulpride, during adolescent social defeat prevented the reduction in adult mPFC DA levels (Watt et al., 2014). However, no studies have investigated the impact of preventing stress-induced mPFC DA hypoactivity on locomotor responses to psychostimulant drugs.
Figure 5-1. Simplified working model for Adolescent CVSS effects on nicotine-induced locomotion. (A) Dopamine (DA) inhibits glutamatergic projection neurons in the medial prefrontal cortex (mPFC) via D2 receptors on GABAergic interneurons. (B) Adolescent stress suppresses mPFC DA disinhibiting cortical-accumbens projection neurons. Synaptic plasticity at NAc glutamate synapses enhances nicotine-induced DA release in NAc. Augmented NAc DA transmission would then increase locomotor activity in response to a nicotine injection.
A series of future experiments should evaluate the proposed working model (Figure 5-1). First, it would be necessary to demonstrate the D2 receptors mediate CVSS effects on nicotine-induced locomotion. Watt et al. (2014) administered intra-mPFC infusions of amisulpride (D2 antagonist) prior to each of 5 adolescent social defeat exposures. This manipulation is not feasible using the CVSS paradigm (due to chronic 24 hour stress exposure). However, control males that received intra-mPFC infusions of quinpirole (D2 agonist) during adolescence would be expected to exhibit increased locomotor responses to nicotine as compared to saline-treated controls and saline/amisulpride-treated CVSS males. If intra-mPFC quinpirole did not influence nicotine responses in CVSS males, then results may suggest there is ceiling effect on D2 signaling, where by maximum inhibition of DA synthesis has occurred, in the adolescent mPFC. Alternatively, there could be an additive effect of quinpirole treatment on nicotine-induced locomotion for CVSS males. This dose-dependent enhancement in the effects of adolescent D2 activation would further support a relationship between mPFC DA and locomotor responses to nicotine.

Once a link between mPFC D2 activity and nicotinic-induced locomotion has been identified, additional experiments would be required to assess causal mechanisms. Recent advances in optogenetics have provided a powerful tool for dissecting the roles of discrete neural circuits in behavior. For example, a viral vector expressing channelrhodopsin-2 (ChR2) under the control of the CaMKIIa promotor can be used to selectively activate glutamatergic terminals in the NAc (Bagot et al., 2015; Britt et al., 2012). CaMKIIa is specifically expressed in glutamatergic neurons so the viral infection
will result in ChR2 expression in only these neurons within an injected brain region (Basu et al., 2008). When injected into the mPFC, the terminals of NAc-projecting glutamate neurons will express ChR2 allowing the experimenter to optically evoke NAc glutamate release.

In the proposed experiment, nicotine-induced locomotion would be examined following optical stimulation of mPFC glutamate terminals in the NAc of CVSS and control males. Specifically, optogenetic low frequency stimulation of ChR2-expressing terminals in the NAc would attenuate mPFC-NAc glutamate transmission by inducing long-term depression (LTD) (Bagot et al., 2015). This manipulation would be expected to reverse the effects of adolescent CVSS on nicotine-induced locomotion i.e., mPFC-NAc LTD would reduce locomotor responses to nicotine in ChR2-expressing CVSS mice relative CVSS mice injected with a control vector that does not express ChR2 (and therefore do not exhibit LTD at mPFC-NAc synapses). As for the control group, nicotine-induced glutamate release in the NAc is also thought to mediate locomotor responses (Lenoir & Kiyatkin, 2013). So, mPFC-NAc LTD might enhance nicotine-induced locomotion in ChR2-expressing control mice, relative to control mice expressing the control vector. Although additional control experiments would be necessary, these results would begin to suggest that enhanced synaptic activity at mPFC-NAc glutamate terminals is sufficient to enhance locomotor responses to nicotine. Taken together, these results would also suggest that stress-induced enhancements in NAc glutamate are evoked by impairments in mPFC D2 signaling.
5.3.2. **Evaluating the impact of adolescent CVSS on additional inbred mouse strains**

Will adolescent CVSS have similar effects on affect-related behavior and nicotine responses in another inbred mouse strain? For example, would C57BL/6J mice, which are characterized by a low-anxiety phenotype and stress-resilience (Jacobson & Cryan, 2007), exhibit increased anxiety and depression-related behavior or altered nicotine responses as were observed in the BALB/cJ males? The inclusion of additional mouse strains could greatly enhance the translational relevance of these studies. Strain-differences in the effects of adolescent social stress could also provide valuable mechanistic insight into the biological and environmental determinants of susceptibility to anxiety and depression-related behavior and nicotine use.

Well known strain differences in stress susceptibility suggest that BALB/cJ mice may be more susceptible to adolescent social stress than are C57BL/6J mice. For example, one study found that BALB/cOlaHSD and C57BL/6JOlaHsd adult males both exhibit depression-related behavior following exposure to chronic social defeat stress. However, these behavioral effects were only observed in BALB/cOlaHSD males following exposure to a less severe version of the social defeat paradigm (Savignac et al., 2011). With respect to adolescent social stress, recent findings from our lab refute this hypothesis (Caruso et al., *in prep*). In these experiments, male and female C57BL/6J mice were studied under the exact same experimental conditions and behavioral testing procedures as described in Chapter 4. Surprisingly, C57BL/6J males exposed to adolescent CVSS exhibited increased anxiety-related behavior on the EPM, but their behavioral or physiological responses to nicotine were unaffected. The results suggest
that adolescent stress effects on affect-related behavior and nicotine responses may not be related to a common underlying biological mechanism as discussed in Chapter 1. The divergent phenotypes exhibited by male BALB/cJ and C57BL/6J mice exposed to adolescent CVSS (Figure 5-2) could also suggest that, in males, genetic background moderates stress effects on a common mechanism.

Inbred C57BL/6 and BALB/c strains exhibit multiple genetic polymorphisms that may contribute to these findings (Jacobson & Cryan, 2007). However, one of the most well characterized genetic variants, of those known to impact affect-related behaviors, between these strains is a single nucleotide polymorphism in the TPH2 gene (C1473G) (Zhang, Beaulieu, Sotnikova, Gainetdinov, & Caron, 2004). TPH2 encodes a brain-specific isoform of tryptophan hydroxylase which is the rate-limiting enzyme for serotonin (5-HT) synthesis (Walther et al., 2003). BALB/cJ mice, which are homozygous for the 1473G allele, exhibit reduced 5-HT synthesis compared to strains that are homozygous for the 1473C allele such as C57BL/6J mice (Cervo et al., 2005; Zhang et al., 2004). This 5-HT deficiency is associated dysfunctional 5-HT receptor signaling as

![Figure 5-2. Working model illustrating sex and strain-dependent effects of adolescent CVSS on anxiety-related behavior and nicotine responses.](image-url)
genetic knockdown of TPH2 function, which caused a phenotype similar to that observed in BALB/cJ mice, resulted in increased anxiety and depression-related behavior, blunted 5-HT$_{1A}$ receptor function, and compensatory upregulation of 5-HT$_{2A}$ receptor levels (Jacobsen et al., 2012). Taken together, these findings suggest that strain differences in 5-HT system contribute to baseline phenotypic differences observed between the “stress-resilient” C57BL/6J and “stress-susceptible” BALB/cJ strains.

Recent evidence suggests that cholinergic activity is elevated in depression and that interactions between cholinergic and serotonergic signaling may be an important component in the pathophysiology of stress-related psychiatric disorders, like anxiety and depression (Hannestad et al., 2013; Mineur et al., 2014; Saricicek et al., 2012). In mice, genetic and pharmacological inhibition of hippocampal acetylcholine esterase (AChE) increased susceptibility to the anxiety- and depression-related behavioral effects of chronic social defeat stress. Fluoxetine, which stimulates hippocampal AChE activity, reversed these effects (Mineur et al., 2013). The anxiolytic and antidepressant effects of reduced hippocampal cholinergic tone can be recapitulated by drugs that reduce α4β2* (* indicates the possible presence of additional subunits) nicotinic acetylcholine receptor (nAChR) signaling such at the partial agonist cytisine (Mineur et al., 2009, 2007). Interestingly, cytisine’s antidepressant-like effects and ability to increase resilience to social defeat stress require hippocampal 5-HT$_{1A}$ receptors (Mineur et al., 2014). It should be mentioned that that these studies were all performed in adult male C57BL/6J mice (Mineur et al., 2009, 2013, 2014, 2007).
The interaction between $\alpha 4\beta 2^*$ nAChR and 5-HT$_{1A}$ receptor signaling is intriguing in light of strain differences between BALB/c and C57BL/6 mice. In particular, 8-OH-DPAT (5-HT$_{1A}$ receptor agonist) increases anxiety-like behavior in BALB/c mice, but has no effect in C57BL/6 mice (van den Buuse, Martin, Holgate, Matthaei, & Hendry, 2007). Additionally, the antidepressant-like effects of chronic fluoxetine treatment in BALB/cJ mice are not dependent on 5-HT$_{1A}$ receptor function (Holick, Lee, Hen, & Dulawa, 2008). The nAChR system also differs markedly between these strains. C57BL/6ByJ mice are more sensitive the pharmacological effects of nicotine and exhibit increased $\alpha 4\beta 2^*$ nAChR binding site density throughout the brain relative to BALB/cByJ mice (Marks, Romm, Campbell, & Collins, 1989; Marks, Stitzel, & Collins, 1989). Taken together, these findings suggest that the anxiolytic/antidepressant properties and stress-resilience induced by cytisine (Mineur et al., 2009, 2014, 2007) may differ, perhaps substantially, between BALB/cJ and C57BL/6J mice.

Future studies could determine whether pharmacological manipulations of $\alpha 4\beta 2^*$ nAChR and 5-HT$_{1A}$ receptors influence the expression of anxiety-like behavior and altered nicotine responses in C57BL/6J and BALB/cJ males following exposure to adolescent CVSS. Based on the work of Mineur and colleagues, we could propose several hypotheses. First, cytisine will reduce anxiety-related behavior in C57BL/6J, but not BALB/cJ, mice exposed to adolescent CVSS relative to cytisine and saline treated control mice. Second, the anxiolytic effects of a subthreshold dose of cytisine will be potentiated by a subthreshold dose of the 5-HT$_{1A}$ receptor agonist 8-OH-DPAT in
stressed C57BL/6J males relative to controls with no effects in BALB/cJ males. Lastly, the anxiolytic effects of cytisine will be abrogated by pretreatment with the 5-HT_{1A} receptor antagonist WAY100,635 in stressed C57BL/6J males relative to controls with no effect in BALB/cJ males.

It is conceivable that cytisine could prevent or reduce the effects of CVSS on locomotor responses to nicotine, HPA response to nicotine, and voluntary oral nicotine intake observed in BALB/cJ male exposed to adolescent CVSS relative to controls. All of these nicotine responses are mediated, in part, by α4β2* nAChRs (Labarca et al., 2001; Matta et al., 1998; Picciotto et al., 1998; Tapper et al., 2004; Wilking, Hesterberg, Crouch, Homanics, & Stitzel, 2010). That being said the influence of 5-HT_{1A} manipulations on these behaviors is more difficult to predict. Nicotine has been shown to stimulate 5-HT release, but the literature primarily implicates 5-HT in nicotine’s effects on anxiety- and depression-related behaviors (Picciotto & Corrigall, 2002).

Together, the results of this study would provide valuable insight into the mechanisms underlying CVSS effects on the divergent phenotypes observed in C57BL/6J and BALB/cJ males (Figure 5-2). If the same manipulation (cytisine treatment), reversed strain-dependent responses to CVSS, then the results would support the hypothesis that stress-induced changes in a common mechanism (the nAChR system) can lead to divergent behavioral phenotypes depending on genetic background.
5.4. Conclusion

The results of this dissertation help to clarify the factors that influence adolescent social experiences on adult HPA activity, affect-related behavior, and nicotine responses. It is important for future studies to further investigate causal mechanisms underlying the stress effects on affect-related behavior and nicotine responses. Additionally, future studies can enhance the translational value of these findings by investing stress effects in different inbred mouse strains. As we have shown, there are several important factors that contribute to the impact of stress on these physiological and behavioral processes. We then attempted to partially control for these sources of variability by developing a novel social stress paradigm and applying it to a novel research question (i.e., factors contributing to comorbid nicotine use and affective disorders). Although we initially hypothesized that comorbidity would be related to some common underlying biological process, our result suggested this may not be the case. There was little relationship between stress effects on affect-related behavior and nicotine responses. Additional research is necessary to determine the mechanisms that underlie these phenotypes and enhance the translational relevance of our findings.
5.5. References


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APPENDIX: Supplemental Information for Chapter 4

Supplemental Materials and Methods

*Computation of social interaction Z-Scores based on behavior in the Social Interaction Test.*

Social interaction Z-scores (SI Z-scores) were calculated as previously described (Anacker et al., 2016) to integrate 1) time spent in the interaction zone during trial 2 (social target present), 2) time spent in the corner zones during trial 2, 3) the ratio of time in the interaction zone during trial 2/time in the interaction zone during trial 1 (social target absent), and 4) the ratio of time in the corner zones during trial 2/time in the corner zones during trial 1. Briefly, Z-scores were calculated for each measure. The interaction zone ratio and time spent in the interaction zone were then multiplied by -1 so that for all measures, a higher Z-score indicates more social avoidance. The final value was then calculated by averaging all 4 scores.

*Assessment of potential associations between affect-related behaviors and nicotine responses.*

Partial correlation matrices, controlling for litter, were constructed for exploratory analyses of potential interrelationships between affect-related behaviors and nicotine responses. Separate matrices were constructed for males and females due to sex-specific effects of adolescent social stress observed in each experiment.
Figure A-1. Correlations for EPM behaviors, SI test behaviors, and nicotine responses in Experiment 1 males. A correlation matrix was constructed to compare potential interrelationships between measures of affect-related behavior and nicotine responses within individuals (N= 15-22). A correlation coefficient is given in each cell of the matrix. For responses that were significantly correlated ($p < 0.05$), the color intensity and size of the circles are proportional to the respective correlation coefficients (red = positive correlation and blue = negative correlation).
Figure A-2. Correlations for EPM behaviors, SI test behaviors, and nicotine responses in Experiment 1 females. A correlation matrix was constructed to compare potential interrelationships between measures of affect-related behavior and nicotine responses within individuals (N= 12-29). A correlation coefficient is given in each cell of the matrix. For responses that were significantly correlated ($p < 0.05$), the color intensity and size of the circles are proportional to the respective correlation coefficients (red = positive correlation and blue = negative correlation).
Figure A-3. Correlations for EPM behaviors, SI test behaviors, and nicotine responses in Experiment 2 males. A correlation matrix was constructed to compare potential interrelationships between measures of affect-related behavior and nicotine responses within individuals (N= 16-18). A correlation coefficient is given in each cell of the matrix. For responses that were significantly correlated (\( p < 0.05 \)), the color intensity and size of the circles are proportional to the respective correlation coefficients (red = positive correlation and blue = negative correlation).
Figure A-4. Correlations for EPM behaviors, SI test behaviors, and nicotine responses in Experiment 2 females. A correlation matrix was constructed to compare potential interrelationships between measures of affect-related behavior and nicotine responses within individuals (N= 21-22). A correlation coefficient is given in each cell of the matrix. For responses that were significantly correlated ($p < 0.05$), the color intensity and size of the circles are proportional to the respective correlation coefficients (red = positive correlation and blue = negative correlation).
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