EARLY ADOLESCENT NICOTINE EXPOSURE HAS LONG-LASTING
EFFECTS ON COCAINE-INDUCED BEHAVIORS IN MICE

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EARLY ADOLESCENT NICOTINE EXPOSURE HAS LONG-LASTING EFFECTS ON COCAINE-INDUCED BEHAVIORS IN MICE

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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January, 2013
DEDICATION

This thesis is dedicated to someone special who influenced my life and made me the way I am today, my mother. She is the reason for this accomplishment, she is my super HERO. She dedicated all her life after my father passed and raised my sister and me. It was her dream for me to get a much better education than she received; I believe, today her dream has come true.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>5HT</td>
<td>serotonin</td>
</tr>
<tr>
<td>AAALAC</td>
<td>Association for Assessment and Accreditation of Laboratory Animal Care</td>
</tr>
<tr>
<td>Ach</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>AMG</td>
<td>amygdala</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CPP</td>
<td>conditioned place preference</td>
</tr>
<tr>
<td>CPA</td>
<td>conditioned place avoidance</td>
</tr>
<tr>
<td>CREB</td>
<td>cyclic AMP response element binding protein</td>
</tr>
<tr>
<td>CS</td>
<td>conditioned stimulus</td>
</tr>
<tr>
<td>DA</td>
<td>dopamine</td>
</tr>
<tr>
<td>ΔFosB</td>
<td>Delta FosB</td>
</tr>
<tr>
<td>DHβE</td>
<td>dihydro-beta-erythroidine</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>HET</td>
<td>heterozygous</td>
</tr>
<tr>
<td>HIP</td>
<td>hippocampus</td>
</tr>
<tr>
<td>hr.</td>
<td>hours</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>mg/kg</td>
<td>milligrams/kilogram</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>NAc</td>
<td>nucleus accumbens</td>
</tr>
<tr>
<td>nAChR</td>
<td>nicotinic acetylcholine receptor</td>
</tr>
<tr>
<td>NE</td>
<td>norepinephrine</td>
</tr>
<tr>
<td>NRT</td>
<td>nicotine replacement therapy</td>
</tr>
<tr>
<td>PFC</td>
<td>prefrontal cortex</td>
</tr>
<tr>
<td>SAMHSA</td>
<td>Substance Abuse and Mental Health Services Administration</td>
</tr>
<tr>
<td>s.c.</td>
<td>subcutaneous</td>
</tr>
<tr>
<td>sec</td>
<td>seconds</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>US</td>
<td>unconditioned stimulus</td>
</tr>
<tr>
<td>VTA</td>
<td>ventral tegmental area</td>
</tr>
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</table>
Nicotine is one of the most commonly used drugs among adolescent populations. Given the fact that adolescence is a unique developmental stage, during which nicotine has long-term effects on future drug-taking behavior, it is essential to understand how early exposure to nicotine during adolescence may affect the abuse liability of other drugs. We hypothesize that repeated exposure to low doses of nicotine in adolescence induces age-specific enhancement of the rewarding effects of several drugs of abuse in the conditioned place preference (CPP) test. Furthermore, we predict that these changes in behavioral responses are mediated by nicotine-induced brain region-specific increases in the expression of ΔFosB, a member of the Fos family of transcription factors, through activation of neuronal nicotinic receptors.

We used mice as a model system to investigate the effects of adolescent nicotine exposure on responses to cocaine, amphetamine, and morphine in adulthood. We found that exposure to nicotine during the early phase of adolescence (postnatal day 28) enhanced cocaine CPP, acute locomotor activity, and locomotor sensitization in adulthood. Our data demonstrate that nicotine priming effects on cocaine are affected by the dose, duration, method of administration, age of exposure, and mouse strain. These data strongly suggest
that nicotine intake during adolescence may cross-sensitize the brain to the rewarding effects of cocaine. A follow-up study was undertaken to determine if this enhancement applies to other drugs of abuse. The repeated exposure to 0.5 mg/kg nicotine (subcutaneous) during early adolescence resulted in significant enhancement of amphetamine and morphine preference in a CPP test, but had no effect on the somatic signs of morphine withdrawal. In addition, we investigated the possible neuronal mechanisms underlining enhancements to behavioral responses using both in vivo and in vitro techniques. Our results showed that nicotinic antagonists, with varying subtype selectivity, administered during adolescence prior to nicotine exposure diminished cocaine enhancement in CPP. This suggests that the enhancement of cocaine behavioral responses is mediated by neuronal nicotine receptors (mainly β2* and α7). Finally, studies of ΔFosB revealed significant effects of age and nicotine pre-treatment in nucleus accumbens (NAc), but not in the prefrontal cortex (PFC). Indeed, nicotine pre-treatment was able to significantly increase ΔFosB levels in NAc of early adolescent mice compared to adult mice. This accumulation of ΔFosB persisted for several weeks. Further studies are needed to fully examine the mechanisms of action underlying the observed changes in cocaine rewards.
CHAPTER ONE

General introduction

1.1. Tobacco smoking and nicotine dependence

Despite a multitude of campaigns and advertisements describing the dangerous effects of smoking, it is still an increasing problem in the United States and worldwide. According to the 2011 National Survey on Drug Use and Health, an estimated one in four Americans aged 12 or older, were current (past month) users of a tobacco product (cigarettes, cigars, smokeless tobacco, or tobacco pipes), with the highest rates of smoking occurring in people 18-25 years of age (Substance Abuse and Mental Health Services Administration (SAMHSA), 2012). Moreover, approximately 443,000 persons in the United States die from smoking-related illnesses every year. Most smoking-attributable deaths were due to lung cancer, coronary heart disease, chronic obstructive pulmonary disease, and other airway obstruction (Centers for Disease Control and Prevention (CDC), 2012). This makes smoking by far one of the most prevalent causes of preventable death. Yet, the impact of smoking goes beyond health. It has been estimated to approach $96 billion in direct medical costs annually (CDC, 2010). Unfortunately, the addictive property of nicotine is an obstacle for those with a strong desire to quit. Of those who use and/or are addicted to tobacco and eventually attempt to quit, only about 3–5% remain abstinent for 6–12 months (Hughes et al., 2004).

Current pharmacological treatments that are approved for treating tobacco dependence consist of nicotine replacement therapy (NRT), bupropion (Zyban®), and varenicline (Chantix®). NRTs include the nicotine patch, nicotine gum, nicotine lozenge, nicotine inhaler,
and nicotine nasal spray. Bupropion is an atypical antidepressant. Its primary pharmacological action is inhibition of dopamine (DA) reuptake in the central nervous system (CNS) (Miller et al., 2002), but it is also a non-competitive antagonist at the α4β2* and α3β4* nicotinic acetylcholine receptors (nAChRs) (Slemmer et al., 2000). Varenicline is primarily an α4β2* nAChR partial agonist, but is also an α3β4 nAChR partial agonist, as well as a full agonist at α7 nAChRs (Mihalak et al., 2006). The challenges with existing smoking cessation drugs are modest outcomes with variable success rates (McNeil et al., 2010). In addition, significant adverse side effects, such as gastrointestinal (e.g. nausea, dry mouth) and neurological disturbances (e.g. headache, difficulty sleeping, agitation, and abnormal dreams), have been reported for bupropion and varenicline. Therefore, there is a compelling need for more effective drugs to assist smokers in achieving long-term abstinence.

1.1.1. Smoking among adolescents

Despite the vast amount of information regarding the physical and financial effects of tobacco use, adolescent tobacco use is still a rising problem worldwide, as well as in the United States. Although much progress has been made in the past few years to reduce the prevalence of smoking, adolescent tobacco use still represents one of the major challenges to the future of public health. In fact, in 2011, 44.7% of adolescents reported using a tobacco product at some point in the past month (CDC, 2012).

In recent years, tobacco consumption has expanded outside of traditional products like cigarettes and cigars, to include smokeless tobacco products and hookahs. Novel smokeless products include oral flavored tobacco lozenges, like Ariva®, Snus®, which are sachets, as well as dissolvable tobacco in the form of strips, sticks, and orbs. Hooka is a water-pipe in which
fruit-scented tobacco is burnt using coal and becomes smoke, then inhaled through a hose. These new products are particularly attractive to adolescents and young adults. According to the CDC, approximately 6.1% of high school students were current smokeless tobacco users in 2009 (CDC, 2010). These oral smokeless tobacco products are attractive for a number of reasons. Chiefly, the flavored versions of these products mask the taste and smell typically associated with tobacco, appealing to younger users. Evidence suggests that “taste”, “smell”, and “being different to try” are important determinants of the use of these novel tobacco products (Soldz and Dorsey, 2005). Additionally, deceptive marketing tactics and cheaper prices for these products are also appealing. Together, these factors have contributed to the popularity of smokeless products, especially in young adults (American Lung Association, 2012). These products come in a variety of easy to conceal sizes, with packaging that is as colorful as that seen with candies (e.g. M&M’s and TicTacs), allowing users to individualize their tobacco consumption. Because of the aforementioned marketing, some consumers erroneously believe that these are healthier alternatives to traditional products.

According to the Surgeon General’s Report (2012), over 3,800 teenagers (12-17 years old) begin smoking every day, and of those 3,800, approximately 25% become regular smokers (U.S. Department of Health and Human Services, 2012). Despite the fact that the majority of adolescents report using tobacco products for the first time because of sociocultural factors such as peer pressure, curiosity, and advertising (reviewed in Tyas and Pederson, 1998), once smoking is initiated, cessation is difficult, and smoking is likely to be a lifetime addiction.

Correspondingly, studies have found that the onset of smoking in early adolescence significantly correlates with long-term nicotine dependence (Chassin et al., 1990; Hu et al., 2006). In addition, another study found that students who tried a single cigarette by age 11
remained vulnerable to smoking for up to 3 years (Fidler et al., 2006). Furthermore, several longitudinal studies found that the initiation of smoking during adolescence correlated with greater addiction liability, higher daily consumption, and reduced likelihood of quitting (Colby et al., 2000; Kandel and Chen, 2000; Riggs et al., 2007). Consequently, there is a remarkably strong correlation between adolescent smoking and the subsequent development of long-lasting tobacco addiction.

Many adolescent smokers express the desire to quit and try to do so, but like adult smokers, most have failed (reviewed in Bancei el al., 2007). Research in smoking cessation for adolescents has gained significant attention within the last few decades, yet few well-designed smoking cessation studies have been conducted with adolescents. Interventions are typically distinguished as either intended for prevention or cessation. A confound that remains in the research is the imprecise boundary between young smokers, experimenters, and 'potential' smokers. Therefore, some interventions are aimed towards combining both approaches (Grimshaw and Stanton, 2006). To date, no smoking cessation medications are approved by the United States Food and Drug Administration (FDA) for use in people under 18 years old, yet NRT and bupropion have been studied to a limited extent in the adolescent population to determine the efficacy, safety, and tolerability (Moolchan et al., 2005; reviewed in Karpinski et al., 2010). Much work remains to be done in the research of smoking cessation medications, and in pharmacological and non-pharmacological areas of treatment in adolescents.

1.1.2 Nicotine and nicotinic acetylcholine receptors

Nicotine, the primary addictive component in tobacco, mimics the effects of the endogenous transmitter acetylcholine (ACh) by binding to nAChRs in the periphery and central
nervous system. nAChRs belong to the superfamily of ligand-gated ion channels that includes GABA<sub>A</sub>, glycine, and 5-HT<sub>3</sub> serotonin receptors. These ionotropic receptors have a pentameric structure consisting of five membrane-spanning regions around a central ion channel. Nine nAChR subunits are expressed in the central nervous system, including α2-α6, α7 homopentamers, and β2-β4, which make possible a much larger variety in subtype composition within the brain, leading to a much larger range of pharmacological effects in response to nicotine (Dani and Bertrand, 2007). These subunits have varying degrees of permeability to Na<sup>+</sup>, Ca<sup>2+</sup>, and K<sup>+</sup> (Gotti et al., 2006; Wonnacott et al., 2005). There are two predominant types of nAChRs in the brain: α4β2* heteromers which have two binding sites (* is conventionally used to denote the possible assembly with other subunits) and α7 homomers, which have five binding sites (Changeux et al., 1998). The α4β2* nAChRs are thought to have the highest affinity for nicotine (McGehee and Role, 1995; Picciotto et al., 1995), while α7 nAChRs are thought to have a lower affinity for nicotine compared to α4β2* nAChRs.

Nicotine exerts its rewarding and reinforcing effects by activating ventral tegmental area (VTA) dopaminergic neurons directly via stimulation of β2* nAChRs (α4β2* and/or α6β2*). Stimulation of β2* nAChRs lead to increased rates of dopaminergic neuron firing and subsequent increases in dopamine release in the nucleus accumbens (NAc) (Pontieri et al., 1996). Similarly, stimulation of α7 nAChRs on glutamatergic neurons secondarily stimulates innervated dopamine cells. Previous pharmacological evidence revealed that both systemic and local intra-VTA injections of DhβE, a selective β2* antagonist, blocked nicotine self-administration and conditioned place preference (CPP). Similarly, mice lacking the β2 subunit did not exhibit nicotine CPP (Walters et al., 2006) or nicotine self-administration (Pons et al., 2008) compared to wild-type counterparts. In addition, re-expression of β2 subunits in the VTA of β2-knockout
mice reinstated nicotine-induced DA release (Maskos et al., 2005) and nicotine intravenous self-administration (Pons et al., 2008). Pons et al. (2008) reported that the re-expression of either the α4 or α6 nAChR in the VTA reinstated nicotine self-administration, consistent with expression of α4β2 and α4α6β2* nAChRs in the VTA. This confirmed the role of β2* nAChRs in combination with both α4 and α6 subunits in reinforcing the properties of nicotine. Interestingly, adolescent rodents consistently express greater numbers of α4β2* nAChRs compared to the same brain regions of adult rats (Doura et al., 2008). Therefore, one can speculate that greater numbers of α4β2 nAChRs in adolescents may contribute to greater reinforcing effects of nicotine in that age group.

The precise role of α7* in nicotine reward and reinforcement behavior remains unclear. Local re-expression of α7 in the VTA did not reinstate intravenous self-administration in α7 knockout mice (Pons et al., 2008). Furthermore the α7 antagonist, methyllycaconitine, had no effect on nicotine self-administration in rats (Grottick & Higgins, 2000) or nicotine-induced conditioned place preference (Walters et al., 2006). Recent data suggest, however, that α7 nAChRs may modulate, rather than mediate, nicotine reinforcement. Indeed, blocking α7 nAChRs in NAc or anterior cingulate cortex in the rat increased the motivation to self-administer nicotine, whereas infusion of a selective α7 agonist decreased motivation, as measured using a progressive ratio schedule (Brunzell and McIntosh, 2012).

1.1.3 Molecular and pharmacological mechanisms involved in drug dependence

Drug addiction can be defined as compulsive drug-seeking and drug-taking behaviors despite negative consequences. While abused drugs have different pharmacological actions, the majority of commonly abused drugs affect similar reward circuitry in the brain; specifically, the
mesocorticolimbic dopamine system and the enhancement of its activity (Wise, 1996). This system consists of dopaminergic neurons in the VTA and their axonal projections to limbic structures and cortical areas. The mesolimbic pathway includes the amygdala, ventral pallidum, hippocampus, and NAc, while the mesocortical pathway includes the prefrontal cortex (PFC), orbitofrontal cortex, and anterior cingulate.

There is an abundance of evidence supporting the role of the dopamine system in the reward properties of various drugs of abuse. *in vivo* microdialysis in animals with lesions in NAc demonstrated a loss of drug utilization (Robinson and Berridge, 2001; Nestler, 2004). Some researchers in the addiction field suggest that addiction is primarily derived from repeated stimulation of the mesolimbic dopamine system, leading to significant alterations in reinforcement mechanisms and motivational states (Nestler, 2004).

Essentially, cocaine, amphetamine, morphine, and other abused drugs have been shown to increase extracellular DA in NAc (Self and Nestler, 1995). Cocaine, the prototypical psychostimulant, exerts its behavioral and reinforcing property through inhibiting the reuptake transporters for dopamine (DAT), norepinephrine (NET), and serotonin (SERT), leading to elevated signaling of all three neurotransmitters. Specifically, cocaine binds tightly at the DAT forming a complex that blocks the reuptake of DA. This results in an accumulation of DA in the synaptic cleft, and the subsequent prolonged postsynaptic effect of dopaminergic signaling at dopamine receptors (Goodman et al. 2011). In contrast, amphetamine increases DA at the synaptic cleft by promoting DA release from vesicles (VMAT2), and the reverse transport of dopamine from the presynaptic neuron into the synaptic cleft. Morphine, through activation of mu-opioid receptor, is known to excite dopamine neurons in the VTA by inhibition of
GABAergic inhibitory interneurons, thereby increasing dopamine transmission to NAc (Rezayof et al. 2007).

Another possible elucidation of the long-lasting nature of addiction is thought to be caused, at least in part, by changes in neural gene expression. A main mechanism through which signal transduction pathways influence gene expression is the regulation of transcription factors—proteins that bind to regulatory regions of genes and modify their transcription. Two transcription factors have been strongly implicated in the addictive properties of drugs of abuse: (1) ΔFosB (a truncated splice variant of the FosB gene), and (2) cyclic adenosine monophosphate response element–binding (CREB) protein. It is likely that exposure to drugs alters the circuitry in the brain and induces ΔFosB or CREB, or both, which causes further long-term effects (Nestler et al. 2008; Nestler et al. 2001).

Nicotine, cocaine, amphetamine and other drugs of abuse have been shown to induce the transcription factor ΔFosB after chronic administration (Pich et al. 1997). The precise pathway of ΔFosB activation is unknown. ΔFosB is a member of the Fos family including c-Fos, FosB, Fra1, and Fra2. These other Fos family members are induced rapidly in the striatum in response to acute administration of psychostimulants, however due to their instability their expression are transient and return to basal levels within hours (Figure 1.1).

An elevated expression of ΔFosB accompanies repeated exposure to drugs of abuse, especially in brain areas associated with reward and motivation such as NAc. Indeed, mice overexpressing ΔFosB in NAc showed enhanced sensitivity to both the acute locomotor and rewarding effects of cocaine (Kelz et al. 1999) and enhanced morphine CPP (Zachariou et al. 2006). ΔFosB is a suitable candidate for causing long-term plasticity effects in particular developmental changes: (1) it gradually increases after repeated exposure to drugs of abuse (2)
its unique long term stability can persist for several days to weeks following stimuli (Figure 1.1) (Chen et al. 1997; Nestler et al. 2008).
Figure 1.1. Scheme showing the gradual accumulation of ΔFosB versus the rapid and transient induction of other Fos family proteins in response to drugs of abuse

(A) Several waves of Fos family proteins (comprising c-Fos, FosB and ΔFosB (33 kD isoform) are induced in nucleus accumbens and dorsal striatal neurons by a single cocaine exposure. (B) A gradual increase in the total levels of ΔFosB isoforms with repeated stimuli during a course of chronic cocaine administration (twice daily) (adapted from Nestler et al. 2008).
Daily exposure to drugs of abuse over a period of time is necessary to increase FosB. Recently, Soderstrom et al. (2007) showed an increase of FosB within NAc and hippocampus of both adolescent and adult rats nicotine exposure (0.4 mg/kg/day from days 34 to 43 and 60 to 69, respectively). Furthermore, this increased ΔFosB expression persisted for weeks. Interestingly, in early adolescent mice a single injection of cocaine or amphetamine for seven days was reported to upregulate ΔFosB in the nucleus accumbens, but did not produce the same response in adult mice (Ehrlich et al. 2002). A limitation of the two studies mentioned previously was that correlations of the increases in ΔFosB to behavioral outcomes were not conducted.

Similarly, increased transcription of the CREB gene accompanies the upregulation of the cAMP pathway known to occur in specific brain regions after repeated drug intake. Induction of the transcription factor CREB has been linked to an increase in the expression of tyrosine hydroxylase (Piech-Dumas and Tank 1999) an enzyme critically involved in the formation of dopamine. Several studies have demonstrated a correlation between nicotine administration and CREB. The activation of CREB is necessary for nicotine rewards in adult mice as measured by conditioned place preference testing (Walters et al. 2005). Another study shows that chronic nicotine administration withdrawal in rats decreases CREB, phosphorylated CREB, and CRE-DNA binding in the cortex and amygdala (Pandey et al. 2001). While the precise role of CREB is not yet determined, it is clear that it is involved in both nicotine reward and withdrawal.

1.2 Adolescent development

Adolescence is the final developmental period leading to adulthood. It is approximately 12 to 18 years old in humans, and 28 to 60 postnatal days in rodents (Spear 2000; Laviola et al. 2003). During this critical period, the transition occurs from a fully dependent child to an
independent adult. This transition involves many changes in a variety of areas, including physical growth, cognition, social skills, and physiology. This developmental maturation allows the individual to reach independence from parental care. Puberty precisely refers to physiological changes associated with sexual maturation. The boundaries for adolescence, however, are less precisely defined, and include both psychological and social factors (Spear, 2000). The adolescence stage in humans is defined by certain behavioral changes, including increases in social interaction, risk-taking activities, and novelty or reward seeking behaviors relative to adults. Similarly, adolescent rodents exhibit increased novelty-seeking behavior and risk-taking (reviewed in Laviola et al. 2003). In a two-bottle choice paradigm, only early adolescent mice showed a significant increase in preference for oral nicotine (compared to water), whereas this was not seen for mice exposed during either late adolescence or adulthood (Adriani et al. 2002). These psychological factors conceptualize adolescence as a high-risk period for smoking and substance-use initiation.

Although many studies support the idea of the involvement of social and psychological factors underlying the susceptibility of adolescents to smoking behaviors, there is evidence that the basic biological differences between the adult and adolescent brain also contribute to the unique susceptibility of adolescents to smoking. In fact, the adolescent brain is unique and exists in a state of transition as it undergoes marked maturation that may play a role in subsequent drug abuse (Spear, 2000). An adolescent brain is anatomically and neurochemically different from that of an adult brain, with the adult brain being approximately 10% larger than an adolescent brain. Human magnetic resonance imaging (MRI) images have shown a linear increase in white matter and an inverted U-shaped change in gray matter volume. Consistent with gray matter changes, the number of synaptic connections increases during early adolescence, and rapidly
declines in late adolescence (Giedd, 2004). The adolescent brain goes through an increase in myelination and synaptic pruning to allow more efficient neural signaling. It has been predicted that on average, as many as 50% of synapses are lost during adolescence. One reason for synapse elimination is to decrease unnecessary excitatory stimuli to the brain, since many of the synapses in adolescence are excitatory (Rakic et al. 1994). Subcortical limbic structures important for emotional processing, such as midbrain dopamine areas, NAc, dorsal and ventral striatum, and amygdala, experience a major developmental boost around the onset of puberty. In contrast, development of the frontal cortical areas of the brain, which are responsible for cognitive control over behavior, depends upon age and experience, and continues throughout adolescence and into adulthood. These structural changes in brain development may be responsible for characteristic adolescent traits, such as uncontrollable mood swings, impulsivity, risk taking, and peer-directed social interactions.

Moreover, the adolescent brain shows remarkable alterations in neurotransmission. Distinctively, the mesocorticolimbic dopamine system goes through significant modeling during adolescent periods. The balance between mesocortical and mesolimbic dopamine systems varies among species (Spear, 2000). These developments are responsible for the integration of the external environment with internal drives to produce motivated behavior (Chambers et al. 2003). During adolescent periods, the decline in PFC volume is evident in humans (Sowell et al. 1999) as well as rats (Van Eden et al. 1990). Moreover, there is a decrease in the density of spines on pyramidal cells in the human PFC (Mrzljak et al. 1990). Dopaminergic innervation of the PFC increases during adolescence to peak levels well above those seen in earlier or later stages of life (Lewis 1997; Brenhouse et al. 2008). Accordingly, there is a transient increase in the number of dopamine transporters and receptors in the PFC (Akbari et al. 1992; Seeman et al. 1987). These
various studies suggest that adolescence is a unique period of intense neurological development, and many of the ongoing changes during this period may contribute to an intensified vulnerability to nicotine and substance abuse.

1.3 The consequence of adolescent nicotine exposure

1.3.1 Behavioral consequence of teen smoking

Adolescents find tobacco more rewarding, underestimate the risks of smoking, and are more influenced by smoking behavior in their social surroundings (peer pressure, family). A review by Mathers and colleagues (2006) addressed the adult consequences of adolescent tobacco use. The review highlights a number of important links between tobacco use in human adolescence and subsequent developmental problems in adulthood, including effects on later tobacco, alcohol, cannabis, illicit drug use and mental health. Compared with nonsmokers, early smokers at grade 7 were more likely to experience academic difficulties as well as severe cases of substance abuse in the future with binge drinking and hard drug use (Ellickson et al. 2001). Furthermore, teens with a history of cigarette use by age 16 were over nine times more likely to begin heroin use by age 32 (Johnson et al. 1995). Given the propensity to drug use later in life it is vital to understand the behavioral and neurochemical processes that occur when adolescents are exposed to nicotine.

1.3.2 Behavioral consequence of nicotine exposure in animals

Rodent studies show that nicotine has greater positive effects (reward) in adolescents than adults, whereas the negative effects (withdrawal) associated with nicotine are less intense in adolescents
(O’Dell, 2009; Kota et al. 2007). Although these studies focused on the short-term effects of nicotine in adolescents, the long-term consequences of nicotine exposure during adolescence are beginning to be more understood. For example, rats first exposed to nicotine during adolescence self-administer more nicotine than rats exposed in adulthood, and these differences in nicotine intake persist into later age (Levin et al. 2003). Similarly, mice exposed to nicotine for a week during early adolescence showed an increase in the rewarding effects of nicotine in the CPP test later in adulthood (Kota et al. 2009), suggesting the uniqueness of this phase. Consistent with data in humans, adolescent nicotine exposure has been shown to affect cocaine and alcohol preferences and sensitivity in rodents (Collins and Izenwasser 2004; Kelley and Middaugh 1999; Kelley and Rowan 2004).

McQuown et al. utilized intravenous pre-treatments containing low doses of nicotine in adolescents over a four-day period (McQuown, 2007). This nicotine exposure resulted in an enhanced cocaine-sensitization response in adolescence. However, the study did not investigate if the priming effects of nicotine persist until later in adulthood. Correspondingly, rats given nicotine at postnatal day 35 for 10 days showed enhancement of cocaine-induced rewards using a CPP paradigm in adulthood (McMillen et al. 2005). It has also been shown that exposure to nicotine in adolescent rats for seven days led to enhanced sensitization to cocaine compared to rats exposed only to saline (McQuown, 2009). In contrast, another study found that C57BL/6J mice demonstrated a decline in cocaine-induced preferences, as measured by CPP after 25 days of nicotine exposure in adolescent mice (Kelley and Rowan, 2004). Although the effects of adolescent nicotine pre-exposure are fairly robust, they appear to be parameter-dependent, such as dosage and exposure protocols. It is therefore possible that these confounding findings are due to differences in parameters or methodologies.
In conclusion, rodent studies show that adolescence is a period of heightened sensitivity to nicotine reward, as well as decreased sensitivity to nicotine's withdrawal and aversive effects. This suggests that smoking in adolescence may promote rapid acceleration to nicotine dependence. Therefore, the vulnerability to the reward effects of nicotine during adolescence may be explained by adolescent brain development. Structural and functional MRI data show earlier maturation of reward systems, and much slower development of prefrontal cognitive control. Taken together, it seems that the adolescent brain is more sensitive to the effects of nicotine than the adult brain, leading to faster progression to nicotine addiction in adolescence than adults.

1.3.3 Neuronal adaptations after nicotine exposure during adolescence

There is substantial evidence from both human and animal studies that nicotine exposure during adolescence disrupts the normal process of the developing brain. For example, in rats, persistent deficits in cognition have been reported after continuous infusion of nicotine during adolescence (reviewed in Dwyer et al. 2009). Also, exposure to nicotine during adolescence may specifically affect the limbic circuitry, producing enhanced vulnerability to nicotine addiction, increased impulsivity, and mood disorders. Counotte and colleagues (2011) reported that rats that underwent repeated exposure to low doses of nicotine during adolescence (PND34-34) led to accuracy reduction in stimulus detection in visuospatial attentional tasks, and an increase in premature and time-out responding. These data showed reduced attention and increased impulsivity that resulted from exposure to a low dose of nicotine. Similar treatment regimens in adult rats did not yield the same behavioral effects, suggesting that nicotine affects normal adolescent brain maturation (Counotte et al. 2011). Additionally, in rats, acute nicotine administration altered neurochemical responses in limbic circuitry in an age- and region-specific
manner. Using in vivo microdialysis, Shearman et al. (2008) reported that acute nicotine significantly induced greater increases in extracellular levels of DA, 5-HT and their metabolites in NAc of adolescent compared to adult rats. These studies in rats suggest that different neuronal adaptations can occur following repeated exposure to nicotine during early developments that influence diverse signaling pathways and circuits well into adulthood (Slotkin, 2002).

Adolescent nicotine exposure leads to acute and longer lasting increases in the levels of α4β2* nicotinic receptors (Abreu-Villaca et al. 2003; Doura et al. 2008), and the function of brain regions, such as the hippocampus, cortex, and striatum in both mice and rats (Kota et al. 2009). In addition, α4β2* nAChRs appear to be selectively upregulated via posttranslational mechanisms (Miwa et al. 2011). Using [3H] cystine, a ligand that binds selectively to α4β2*, Trauth et al. (1999) reported regional and gender-selective effects of adolescent nicotine exposure. In this two-week continuous infusion protocol, upregulation and long-term persistence of nAChR in the midbrain of males, and potential hippocampal cell damage in female rats, differed substantially between adolescents and adults. It was also found that exposure to nicotine during adolescence in an intravenous self-administration protocol, but not a similar exposure in the adult period, increased mRNA expression of dopaminergic neurons expressing the nAChR subunits α5, α6, and β2 subunits in rats (Adriani et al. 2003). Furthermore, α4β2 nAChR upregulation was reported in the adolescent medial PFC shortly following nicotine exposure. This upregulation was accompanied by an increase in nicotine-stimulated GABAergic transmission in the medial PFC (Counotte et al. 2012). In addition to an upregulation of α4β2 nAChRs in the PFC, the same group found that metabotropic glutamatergic receptors type 2 (mGluR2) were significantly upregulated during adolescent nicotine exposure in the same brain
region (Counotte et al. 2011). Importantly, α4β2 nAChR upregulation is the most common cellular adaptation following chronic nicotine exposure (Dani and Bertrand 2007).

Animal studies have also shown that exposure to nicotine during adolescence induces a more intense change in gene expression in several brain regions than during adulthood. For example, the activity of specific early response genes, such as arc and c-fos, which are used as markers for the functional activation of neurons, was increased in various brain regions after exposure to nicotine in adolescent rats (Leslie et al. 2004; Schochet et al. 2005). In addition, nicotine exposure in adolescent rats led to significant changes in genes involved in various plastic cellular and molecular functions, including signal transduction, cytoskeleton dynamics, and transcription, that could initiate long-term structural and functional neuroadaptations (Polesskaya et al. 2007). Importantly, short-term and long-term changes in gene expression unique to the adolescent rat in the VTA were reported after chronic infusion of nicotine. Some of these gene changes associated with neuronal structure and function, such as long-term potentiation and circadian rhythm, persisted into adulthood (Doura et al. 2010).

1.4 Studying the effect of adolescent nicotine exposure in animal models

While no animal model of smoking fully imitates the human adolescent smoker, adolescent animal models do permit examination of certain aspects of nicotine addiction. Limitations of these models often result from difficulties in deciding appropriate age correlations, as well as determining neurobiological correlations. Based on similarities in the physical, social, and biological development of both rodents and humans, three developmental phases of rodent adolescence have been identified: early-(PND 21-34), mid-(PND 35-46), and
late-(PND 47-59) adolescence (Spear, 2000). Given that there are some species differences, the age-limits of these phases are not strict, but rather, are indicative.

Emerging evidence suggests that adolescent humans and rodents experience many similar behavioral and neural changes as they progress to adulthood. For example, human adolescents exhibit an increase in risk-taking behavior, such as drunk driving and using of illegal drugs (Arnett, 1992). Correspondingly, adolescent mice have been noted to exhibit a higher degree of novelty-seeking behaviors compared to adults (Adriani et al. 1998). Similar to behavioral consistencies among adolescent humans and rodents, neural alterations in humans and rodents appear to have correlations. For example, there is a high degree of PFC remodeling noted in both human adolescents (Jernigan et al. 1991) and in rats (van Eden et al. 1990). Moreover, dopamine D1 and D2 receptor levels peak and then decline over adolescence (Gelbard et al. 1989; Teicher et al. 1995; Andersen and Teicher 2000) in both species. Accordingly, brain development during adolescence is in many ways similar between humans and rodents, which validates the use of rodents as appropriate models for human adolescent research.

Yet, designing an appropriate animal model for adolescent nicotine exposure is a challenging proposition, as there are considerations of proper dose, route, and frequency of administration. The pattern of adolescent smoking is different from that of adults, as it occurs in stages and usually involves repeated, albeit irregular, use over an extended period. There are three main patterns of tobacco smoking that is prevalent in adolescent smokers. Some begin usage with three to four cigarettes per week as a phase, and then quit. Some are occasional smokers, in that they smoke a couple of times per month or year. The third group continues to smoke and progress to daily usage, and then dependence. The smoking pattern in adolescence is further complicated by the fact that it is affected by specific events, such as parties and
weekends. Other challenges include differences in the kinetics between mice and humans, with kinetics being faster in the mice, as well as differences in the life cycles, with mice living about a year and humans living for decades. Additionally, only nicotine was examined in the aforementioned studies, which leaves out the myriad of other chemicals that are present in tobacco products, such as acetaldehyde. All of these factors need to be considered when designing, executing, and evaluating mice studies for potential human applications.

There are several methods for delivering an appropriate nicotine dose in mice that mimics the corresponding plasma level in human adolescents as closely as possible. It can be delivered through systemic injection (Kota et al. 2007), orally (via gavage or drinking water), and via a mini-pump or intravenous infusion. When evaluating the use of a particular animal model, it is necessary to consider the phenotype and onset of exposure. Correspondingly, the scope of the research question must also be taken into account. For example, when studying the effect of using a NRT, like a nicotine patch, a subcutaneous mini-pump, which releases a slow and constant infusion of nicotine, can provide a good model for dose delivery. Subcutaneous injection was used for the majority of our studies due to the narrow time frame of adolescence mentioned previously (early adolescence is a ten day period) in mice.

Caution is needed when comparing the resulting experimental effects with mini-pump and injection delivery of nicotine, as those approaches are forms of controlled drug exposure, which neither models daily variations in nicotine levels nor the effects of the many other constituents of tobacco products. Similarly, oral administration of nicotine (via drinking water) provides limited control of the actual dosage received, as it is affected by first-pass metabolism, which leads to variable absorption. Current challenges include translating data from animal
models to humans, and identifying what makes some individuals particularly vulnerable or resistant to nicotine dependence.

1.5 Mouse models of reward and reinforcement

In humans, drugs of abuse function as rewards, and as such, their repeated use is strengthened to a degree meeting the DSM-IV criterion for abuse and dependence. A great deal of effort has gone into adapting appropriate animal models to assess the reinforcing and reward-like properties of drugs of abuse. An increasing number of studies have used the CPP test to assess the rewarding effects of appetitive substances or drugs in animals. In CPP, conditioning involves an animal (mouse) receiving repeated access to the stimulus (unconditioned stimulus; US) in one context (conditioned stimulus; CS) and to saline in the other context. With only one of two distinct sets of environmental (contextual) cues paired with the stimulus of interest (e.g., drug, food). The contextual cues may differ along several stimulus dimensions such as floor, wall color, or odor. Following conditioning, a choice test occurs in which animals receive unrestricted exposure to both contexts in the absence of the US. Presumably, the learned association between the context CS and the US result in animals spending more time in that context. In CPP, an apparent increase in time spent in the paired context relative to a control value is taken as evidence that the US was rewarding. This model is an example of Pavlovian conditioning (Cunningham, 1998). The major focus in this dissertation is to assess the reward-like effects of several drugs of abuse in rodents.

Unlike self-administration (operant paradigm), a CPP test does not directly measure drug reinforcement (defined as strengthen stimulus and/or increases the probability of a class of behaviors); rather, it is a measure of reward-like behavior (which studies the appetitive nature of
a given stimulus). Because of the narrow span of adolescence in rodents, and additional technical challenges, it is extremely difficult to train and establish self-administration in mice within the adolescent window. Moreover, the CPP approach is advantageous because it has a short duration, does not stress the animal with surgery or extensive training, and allows for simultaneous testing of locomotor activity while conducting CPP. The model is adaptable to a variety of species, and tests the animal in a drug-free state. CPP is sensitive to both reward and aversion. Specifically, as established throughout the literature, there is a reasonable concordance between drugs that produce a CPP and drugs that are self-administered (Bardo and Bevins, 2000) such that data from this method compliments self-administration data. Even the reward-like effects of drugs, like buspirone, that are not self-administered will result in CPP (Bardo and Bevins, 2000). Several reports have concluded that cocaine, morphine, amphetamine, and nicotine are able to induce CPP in rodents over a wide range of doses, and with various routes of administration (Le Foll and Goldberg, 2005; Grabus et al., 2006; Walters et al. 2006). In the past few years, there have been striking and exciting developments in regard to the use of the CPP paradigm (reviewed in Tzschentke, 2007), such as an increase in the use of genetically modified animals in addiction research, and the adaptation of extinction–reinstatement procedures for place conditioning. Furthermore, the CPP model is well-established in our laboratory.

There are confounding factors for the interpretation of place preference results. Some of the limitations include locomotor activity changes after drug conditioning, novelty-seeking behavior on the test day, and the tendency of the animals to have contextual preferences. However, extensive work with outbred ICR mice over the years in the CPP test showed that this propensity for contextual preference is rare in this strain, and any animal that does show more than 65-70% preference in the pretesting phase will be excluded from testing. To account for
locomotion behavior, we monitor locomotor activity in the CPP boxes during our studies. Additionally, the boxes utilized are three-chamber compartments (with a central compartment), which limits the impact of novelty-seeking behavior on test day.

1.6 Significance of project

Cumulative epidemiological data linked early smoking initiation with heavier daily consumption and a higher prevalence of nicotine dependence in adulthood (Colby et al., 2000; Kandel & Chen, 2000). Furthermore, adolescent smoking is a risk factor for future drug and alcohol abuse (Kandel et al., 1992; Levine et al., 2011). According to the Substance Abuse and Mental Health Services Administration (SAMHSA) (2009), among those adults aged 18 to 34 who had used cocaine at least once, 90.4% had smoked cigarettes before beginning to use cocaine, and 4.7% began using both drugs at the same age. Breslau and Peterson reported similar results, showing that individuals under the age of 15 who smoked cigarettes were 80 times more likely to use illegal drugs compared to those who didn’t (Breslau & Peterson, 1996). Correspondingly, Johnson et al. (1995) found that early smokers at Grade 7 were more likely to experience severe types of substance use (binge drinking and hard drug use) later in life, as compared to nonsmokers. Furthermore, teens with a history of cigarette use by age 16 were over nine times more likely to begin using heroin by age 32 (Johnson et al., 1995). Unfortunately, the correlational nature of epidemiology makes it hard to determine a causal effect.

The new challenge is to better understand the subsequent effects of smoking in adolescent populations. It is critical to research developmental changes resulting from adolescent tobacco consumption so that we can understand the ways in which adolescents are particularly vulnerable to nicotine dependence and susceptible to addiction. The research in this dissertation will focus on the long-term relationship between nicotine exposure during
adolescence and behavioral and molecular consequences in adulthood. The benefit of this type of research is to advance the understanding of the potential long lasting effects of adolescent tobacco use especially on subsequent drug use.
1.7 Hypothesis

We hypothesize that repeated exposure to low doses of nicotine in adolescence induce an age-specific enhancement of the rewarding effects of several drugs of abuse in the CPP test. Furthermore, we predict that these changes in behavioral responses are mediated by nicotine-induced brain region-specific increases in ΔFosB expression through activation of neuronal nicotinic receptors.

1.8 Dissertation objectives

The research in this thesis focuses on the impact of adolescent nicotine exposure on the subsequent behavioral response toward drugs of abuse, specifically cocaine, amphetamine, and morphine. Based on preliminary data and previous literature, we hypothesized that adolescent mice exposed to low doses of nicotine would demonstrate increased sensitivity during adulthood to commonly abused drugs compared to mice exposed in adulthood.

Our first specific aim was to establish and characterize a mouse model to investigate the impact of nicotine exposure during adolescence on subsequent behavior in adulthood utilizing CPP response to cocaine. The data from this study fully characterized nicotine exposure parameters in mice using different doses, routes, and exposure lengths in the same outcome test. Consequently, the results from these experiments were used to identify one treatment regimen that will be used in our remaining studies.

The second specific aim was to examine the impact of age of exposure on enhanced cocaine preference during adulthood after adolescent nicotine exposure. Using the same administration model, we also assessed other behavioral effects of cocaine, such as locomotor activation and locomotor sensitization, after repeated exposure.
The third specific aim was to examine whether adolescent nicotine exposure effect on cocaine reward generalizes to other commonly abused drugs, such as morphine and amphetamine. The data from this study will give insight into the possible pathway affected by adolescent exposure to nicotine.

Our fourth and final aim was to elucidate the neuronal mechanisms underlining the enhanced behavioral responses by using both in vivo and in vitro techniques. We first established if nicotinic receptors mediate nicotine-induced sensitization of cocaine’s behavioral effects. We then investigated ΔFosB accumulation as one possible underlying mechanism for the behavioral changes resulting from early adolescent nicotine use.
2.1 Introduction

Most studies in the literature focus on how adolescent exposure to nicotine effects outcomes during adolescence, or how adult nicotine exposure effects outcomes during adulthood. On the other hand, there are few reports on the long-term effects of adolescent nicotine exposure on potential drug abuse and subsequent dependence later in life. Studies in rats have demonstrated that short-term treatment with low doses of intravenous nicotine during early adolescence enhanced intravenous cocaine self-administration (McQuown et al. 2007) and cocaine locomotor sensitization later during adolescence (McQuown et al. 2009). It is unknown from these studies if adolescent nicotine exposure would have altered behavioral responses to cocaine or other abused drugs in adulthood. One study by Kelley and Rowan (2004) examined mice that were exposed to nicotine in adolescence, and then measured the behavioral response to cocaine in adulthood. Data from this study showed that adolescent nicotine treatment resulted in a decrease in cocaine preference in the CPP test, and an increased response to cocaine’s locomotor activating effects when tested after a 28-day drug-free period. However, in this study, the investigator used a very high dose of nicotine (3 mg/kg twice per day) throughout the adolescent period.

It is clear that a number of factors may be responsible for the differences between these studies, such as route of administration, drug dosage, length of exposure, and timing of the testing. Since any of these variables, or a combination thereof, may be responsible for the
difference in results, more comprehensive work needs to be done to establish how the long-term effects of adolescent nicotine exposure may be affected by these variables. To this end, our studies focused on characterizing the influence of these factors in a mouse model of nicotine exposure during adolescence on subsequent cocaine responses in adulthood as demonstrated in the CPP test, which will add to the body of knowledge in this field. Specifically, our study addressed parameters that have not been focused on in other studies, such as dose, duration, and route of administration. First, we examined different doses of nicotine, as well as duration of nicotine exposure using subcutaneous (s.c.) injection as route of nicotine administration. Second, we examined the effect of nicotine route of administration on enhanced cocaine preference in the CPP test.

2.2 Materials and methods

2.2.1 Animals

Naïve male ICR mice were obtained from Harlan Laboratories (Indianapolis, IN). We controlled for the litter effect by ordering mice from different litters, which were further divided so that each test group was comprised of mice from different litters. In addition, the unnecessary killing of animals that occurs during culling was avoided. Adolescent mice were shipped immediately after weaning, arrived on postnatal day (PND) 21, and weighed approximately 18-23 g at the start of the experiment. Mice were individually marked for identification, and housed 4 mice per cage. They were allowed to acclimate for 7 days prior to experiments. The mice were handled for 3 days prior to the experiment with unlimited access to food and water. All mice were housed in a humidity and temperature controlled (22°C) vivarium on a 12-h light/dark cycle (lights on at 6 a.m., off at 6 p.m.). Dosing and testing were conducted during the light phase of
the cycle. All animals in the same cage received the same treatment at approximately the same time. Animals were maintained in a facility approved by the American Association for Accreditation of Laboratory Animal Care (AAALAC), and all procedures were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

2.2.2 Drugs

(-)-Nicotine hydrogen tartrate salt [(-)-1-methyl-2-(3-pyridyl) pyrrolidine (+)-bitartrate salt was purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Cocaine HCl was provided by the Drug Supply Program of the National Institute on Drug Abuse (Rockville, MD). Cocaine was injected intraperitoneal (i.p.) at a volume of 10 ml/kg body weight. All drugs were dissolved in sterile saline (0.9% sodium chloride) and prepared fresh before each experiment. All doses are expressed as the free base of the drug.

2.2.3 Drug exposure protocol

Subcutaneous injection, mini-pump, and drinking water were the varied routes of nicotine administration used in this study.

Subcutaneous injection protocol. Mice received nicotine during early adolescence (PND 28-34). Nicotine (0.1, 0.5 and 1 mg/kg) or saline was administered to mice s.c. twice daily with injections approximately 6 hours apart (9:00 am and 3:00 pm) for either acute (1 day) or repeated (7 days) periods. Mice were then housed in their home cages and allowed to mature until they reached adulthood (> PND 70), at which point they were evaluated in the paradigms as described below.
Nicotine infusion via osmotic mini-pumps protocol. Other sets of mice were anesthetized with sodium pentobarbital (45 mg/kg i.p.) and implanted s.c. with Alzet osmotic mini pumps [(models 1007D or 2002); Durect Corporation, Cupertino, CA]. The concentration of nicotine was adjusted according to animal weight and the mini pump flow rate to deliver 2.4 mg/kg/day for 7 days or 24 mg/kg/day for 7 or 28 days.

Nicotine via drinking water protocol. A dose of 30μg/ml of nicotine was added to drinking water and administered for a period lasting 7 days, starting at PND 28 and stopped at the morning of PND 35. The mice were then switched to pure drinking water during the remainder of experiment time. The nicotine solution was changed twice during experiment, prior to the first day and at the end of the third. The control group received drinking water. This 25-50 μg/ml dose range has been shown by our lab and others to be behaviorally active in adult mice (Alsharari et al. 2012; Levine et al. 2011).

2.2.4 Conditioned place preference test

Mice were tested for cocaine induced preference using the CPP test once they reached adulthood (PND70). CPP is a test that reflects a preference for a context due to the repeated association between the context and the drug. The CPP test is well established in our lab and was used for the last several years in other pharmacological and genetic studies as well (Colby, Tiffany et al. 2000, Chen, Millar 1998, Caponnetto, Russo et al. 2012, Hu, Li et al. 2010, RW.ERROR - Unable to find reference:152). The place conditioning chambers and software were purchased from Med Associates (St. Albans, VT). Place conditioning boxes consisted of
two distinct sides (20 cm X 20cm X 20 cm) with a smaller center gray compartment that separated the two sides. Openings from the center compartment allowed access to either side of the chamber.

An unbiased CPP paradigm was utilized in this study according to previously published methods as described in Kota et al. (2007). Briefly, mice were handled for three days prior to the start of the CPP procedure. On day 1, animals were placed in the boxes and allowed to move freely from side to side for 15 min, and time spent in each side was recorded. Days 2-4 were conditioning days, during which the saline group received saline in both the light and dark sides of the boxes whereas cocaine groups received cocaine (10 mg/kg i.p.) in one of the sides and saline in the opposite side. Drug-paired sides were randomized among all groups. On day 5, the same procedure was followed as on day 1 in which the mice were allowed to move freely between all three compartments for 15 min and time spent on each side was recorded. Data are expressed as preference score (time spent on drug-paired side minus time spent on saline-paired side). A positive number indicates a preference for the drug-paired side, whereas a negative number indicates an aversion to the drug-paired side. A number at or near zero indicated no preference for either side.

2.2.5 Measurement of whole blood nicotine levels

To determine whole blood nicotine and cotinine levels a separate group of mice was used, blood samples were drawn by cardiac puncture at 10 min after last nicotine administration (s.c. injection, mini-pumps or oral routes of administration). Whole blood samples were immediately frozen and stored at –80°C until extraction. For extraction, 50 µl of internal standard (ISTD) containing 50 ng of nicotine-d4 and cotinine-d3 in methanol was added to 200
µl of whole blood and mixed. Next, 100 µl of 5M ammonium hydroxide was added to each sample, followed by 2 ml methylene chloride. The samples were mixed for 2 minutes and then centrifuged for 5 minutes at 3000 rpm at 4°C. The organic layer was transferred to a clean test tube. The aqueous phase was extracted twice more with 2 ml of methylene chloride. The organic phases were combined and 500 µl of 25mM hydrochloric acid in methanol was added. Samples were then evaporated to dryness under a gentle stream of nitrogen. They were reconstituted with 100 µl of mobile phase and placed in auto-sample (HPLC/MS/MS) vials for analysis. Samples were analyzed using an Applied Bio system 3200 Qtrap with a turbo V source for Turbolon Spray with a Shimadzu SCL HPLC system controlled by Analyst 1.4.2 software. The chromatographic separation was performed using Hypersil Gold, 3mm X 50 mm, 5 micron (Thermo Scientific, USA). The mobile phase contained 10 mM ammonium formate: methanol (10:90 V/V) and was delivered at a flow rate of 0.5 ml/min. The acquisition mode used was multiple reaction monitoring (MRM) in a positive mode. Transition ions were monitored for nicotine (163>130; 163>117), nicotine-d4 (167>134), cotinine (177>80; 177>98), cotinine-d3 (180>80) and 3-hydroxycotinine (193>80; 193>134). The total chromatographic separation time for each extract injection was 2 minutes. A calibration curve ranging from 12.5 ng/ml to 500 ng/ml was constructed for each compound based on linear regression using the peak area ratios of the drug to its deuterated ISTD. Cotinine-d3 was also used as the ISTD for 3-hydroxycotinine.

2.2.6 Data analysis

For all data, graphs and statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software; San Diego, CA). All CPP results were expressed as mean preference scores ± standard error of the mean. Preference scores were measured in
seconds, and reflected the time spent in the drug paired side pre-conditioning (baseline) subtracted from the time the mice spend in the drug paired side during post conditioning. Statistical analyses of all CPP studies were performed with mixed-factor (one or two-way) ANOVA. Where appropriate, post hoc analyses (Bonferroni) were used to determine which groups were significantly (p < 0.05) different from each other.

2.3 Results

2.3.1 The effect of early-adolescent nicotine exposure on cocaine-induced CPP in adult mice

To assess the effects of early-adolescent nicotine exposure on cocaine-mediated behavioral responses in adulthood, mice were pretreated with nicotine (s.c.) during early adolescence (PND 28), and then allowed to mature to adulthood (PND 70) before CPP testing. One-day exposure protocols (short exposure) with two different doses of nicotine (0.1 or 0.5 mg/kg) were tested (Figure 2.1). All mice conditioned with cocaine in the CPP test during adulthood developed a significant preference (F(1, 36) =104, p < 0.0001) for the cocaine-paired chamber at a dose of 10 mg/kg compared to control mice treated with saline (figure 2.1). Two-way ANOVA analysis revealed no significant effect of adolescent pretreatment [adolescent pretreatment x adult CPP treatment; F(2, 36)=0.12, p =0.8906]. As shown in Figure 2.1, no differences were noted between mice that received one-day of nicotine exposure in adolescence compared to their saline treatment controls. Since the one-day exposure protocol yielded non-significant results, we moved to a 7-day exposure protocol (long exposure), where three different doses of nicotine (0.1, 0.5, or 1.0 mg/kg) were tested. Figure 2.2 shows the results from mice that received nicotine for a 7-day exposure period during adolescence. Pretreatment with nicotine during early adolescence significantly increased the preference for cocaine in adulthood [Two-
way ANOVA (adolescent pretreatment x adult CPP treatment; $F_{(7, 42)} = 30.49, p < 0.0001$) in a dose-related manner. While pretreatment with 0.5 mg/kg nicotine in early adolescence significantly increased the preference for cocaine in adulthood compared to saline-pretreated controls (Figure 2.2), pretreatment with 0.1 and 1.0 mg/kg nicotine in adolescence did not significantly enhance cocaine preference in adulthood compared to saline pretreated controls.
Figure 2.1. The effect in mice of one-day nicotine exposure during early-adolescent on cocaine-induced CPP in adulthood.

(A) Early adolescent mice were injected s.c. with either saline or nicotine (0.1 or 0.5 mg/kg) twice for 1 day, and were assessed for cocaine preference in the CPP test on PND 70. (B) Nicotine pretreatment during early adolescence had no effect on cocaine-induced preference in adulthood. The x-axis shows conditioning treatment in the CPP test. The legend represents the treatment group during early adolescence. Each bar represents the mean ± SEM of seven to eight mice. * p<0.05 from respective saline control.
Figure 2.2. The effect of seven-day nicotine exposure during early-adolescent on cocaine-induced place preference in adult mice.

(A) Early adolescent mice (PND 28) were injected s.c. with saline or various doses of nicotine (0.1, 0.5, or 1 mg/kg) twice daily for 7 days, and were examined for cocaine preference in the CPP test at PND 70. (B) Pretreatment with 0.5 mg/kg nicotine in early adolescence significantly enhanced the cocaine preference in adulthood, while pretreatment with 0.1 or 1 mg/kg nicotine failed to enhance cocaine preference in the CPP test. The x-axis represents conditioning treatment in the CPP test at PND 70. The legend represents the treatment group during early adolescence. Each bar represents the mean ± SEM of seven to eight mice. * p<0.05 from respective saline control. ^p<0.05 from respective saline-cocaine control.
Due to the significant results from mice treated with 0.5 mg/kg nicotine, another group of early-adolescent mice (PND 28) received 0.5 mg/kg nicotine (two injections each day for 7 days), and at PND 70 were then conditioned to various doses of cocaine (1, 5, and 10 mg/kg). Pretreatment with 0.5 mg/kg nicotine during adolescence produced a leftward shift in the cocaine dose-response curve (Figure 2.3), and a subsequent dose of 10 mg/kg cocaine evoked a significant CPP response in adulthood compared to saline controls [two-way ANOVA; adolescent pretreatment x cocaine doses; $F_{(2,36)}=10.64$, $p < 0.05$].
Figure 2.3 The consequence of early-adolescent nicotine exposure on cocaine dose-response curve in the CPP test in adulthood.

(A) Early adolescent mice were injected s.c. with saline or nicotine (0.5 mg/kg) twice daily for 7 days, and were conditioned with differing doses of cocaine (1, 5, or 10 mg/kg) at PND 70. (B) Repeated nicotine exposure in early adolescence produced a leftward shift in the cocaine dose-response curve. The x-axis represents the conditioning treatment in the CPP test. The legend represents the treatment group during early adolescence. Each point represents the mean ± SEM of seven to eight mice. * p<0.05 from respective saline-cocaine.
2.3.2 The effect of nicotine exposure via mini-pump versus drinking water during adolescence on cocaine-induced condition preference in adult mice.

To evaluate the effect of route of administration, naïve mice received either oral nicotine via drinking water (30 µg/ml) or a subcutaneous mini-pump (2.4 or 24 mg/kg/hour for 7 or 28 days) during early-adolescence, and were then evaluated in the CPP test for cocaine reward–like effects during adulthood. Figure 2.4 shows the results from mice that received nicotine via drinking water for 7 days during early-adolescence. One-way ANOVA \( [F (3, 25) =34.71, p < 0.0001] \) showed that pretreatment with 30 µg/ml nicotine in early adolescence significantly increased the preference for cocaine in adulthood compared to tap water pretreated controls.
Figure 2.4. The effect of seven-day nicotine exposure via drinking water during early-adolescence on cocaine-induced CPP in adult mice.

(A) Early adolescent mice were exposed to 30 µg/ml nicotine via drinking water or tap water daily for 7 days. (B) Pretreatment with 30µg/ml nicotine in early adolescence significantly enhanced the cocaine preference in adult mice compared to control. The x-axis represents the conditioning treatment in the CPP test. The legend represents the treatment group during early adolescence. Each bar represents the mean ± SEM of seven to eight mice. * p<0.05 from respective vehicle control. ^ p<0.05 from respective vehicle-cocaine control.
Intriguingly, continuous exposure with various doses of nicotine (2.4 or 24 mg/kg/day for 7 days) via a subcutaneous mini-pump during early adolescence had no significant effects on cocaine-induced preferences \[F(2,29) = 1.1, p = 0.35\] in adulthood compared to saline pretreated controls (Figure 2.5). However, our results demonstrate a significant decrease \[F(3,39) = 1.4, p < 0.05\] of cocaine preference in a duration-related manner following chronic infusion of nicotine (24 mg/kg/day) over 28 days compared to saline-cocaine treatment (Figure 2.6). Continuous exposure to a dose of 24 mg/kg/day of nicotine (via s.c. mini-pump) for 7 days had no significant effect on cocaine CPP \[F(3,39) = 1.4, p = 0.26\] (Figure 2.6). Data from nicotine levels showed that oral and subcutaneous nicotine exposure methods had similar results. However, continuous exposure via the mini-pump resulted in a concentration that was nearly double that from the subcutaneous exposure method for the 24 mg/kg dosage, and a concentration that was nearly three times lower for the 2.4 mg/kg dosage (Table 2.1)
Figure 2.5. The effect of dose of nicotine exposure via seven day mini-pump during early adolescence on cocaine-induced CPP in adult mice.

Osmotic mini-pumps implanted s.c. during early adolescence delivered saline or nicotine (2.4 or 24 mg/kg/day for 7 days). This treatment during early adolescence had no significant effect on cocaine-induced preference in adulthood. The x-axis represents the conditioning treatment in the CPP test. The legend represents the treatment group during early adolescence. Each bar represents the mean ± SEM of five to six mice. * p<0.05 from respective vehicle control.
Figure 2.6. The effect of duration of nicotine exposure via subcutaneous mini-pump during early adolescence on cocaine-induced CPP in adult mice.

Osmotic mini-pumps implanted s.c. during early adolescence to deliver saline or nicotine (24 mg/kg/day for 7 or 28 days). Continuous nicotine pretreatment significantly decreased cocaine-induced preference in adult mice in a duration-related manner. The x-axis represents the conditioning treatment in the CPP test. The legend represents the treatment group during early adolescence. Each bar represents the mean ± SEM of five to six mice. * p<0.05 from respective vehicle control. ^ p<0.05 from respective saline (28 day)-cocaine control.
<table>
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<th>Injection (mg/kg)</th>
<th>Infusion (mg/kg/day)</th>
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<td>Nicotine (ng/ml)</td>
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<td>2.4</td>
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<td>43 ± 8</td>
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Table 2.1 Summary of plasma levels of nicotine after chronic nicotine treatment using s.c. injection, infusion (mini-pumps) or oral routes of administration.

Results are expressed as mean ± SEM of plasma concentrations in ng/ml.
2.4 Summary

In the current study, a mouse model of adolescent nicotine exposure was used to examine the effects of adolescent nicotine exposure on adult cocaine preference in the CPP test. The data showed that a 7 day exposure to nicotine (0.5 mg/kg s.c. twice daily) during early adolescence (PND 28-34) enhanced cocaine CPP in adult male mice (PND 70). This exposure regimen was also able to cause a leftward shift of the cocaine dose-response curve in adult mice (PND 70). While the 7-day s.c. injections of 0.1 or 1 mg/kg nicotine were able to elicit this effect as well, the effect was not as pronounced as the 0.5 mg/kg dose, and was not statistically significant. The effects produced an inverted U-shaped curve, with both 0.1 and 1 mg/kg doses eliciting a diminished response when compared to the 0.5 mg/kg dose. In addition the 1-day exposure protocol of nicotine (0.1 or 0.5 mg/kg) did not affect cocaine CPP in adult mice. This observation suggests that changes in cocaine CPP are dependent upon the pretreatment dose and exposure length. Similarly to the injection protocol, nicotine via drinking water during early adolescence enhanced the adult mouse response to cocaine in the CPP test. However, continuous exposure of nicotine (24 mg/kg/day via subcutaneous mini-pump) significantly decreased cocaine-induced CPP in adult mice, with decrease increasing in magnitude with the duration of the exposure. These results suggest that changes in the sensitivity to cocaine in the CPP test depends on nicotine delivery methods.

In conclusion, our results suggest that adolescent nicotine exposure augments cocaine preference in CPP depending on dose, route, and duration of exposure. It is critical to understand why cocaine preference enhanced in adult mice pretreated with nicotine during early adolescence. A more in depth investigation of these behavioral and molecular mechanisms will be further addressed in Chapters Three and Five.
CHAPTER THREE

Age-dependent effects of low dose nicotine treatment on cocaine-induced behaviors in adult mice

3.1 Introduction

Tobacco is one of the most commonly used drugs during adolescence, and usually precedes the use of other drugs such as cocaine later in life (Kandel et al. 1992). Cocaine and nicotine share common neuronal mechanisms, possibly suggesting that adolescent nicotine exposure can result in alterations to behavioral responses to cocaine. It is of interest to examine how early nicotine exposure may alter cocaine-induced behavioral effects in adult mice. In this set of studies, we extended our first CPP studies to investigate the impact of age and mouse strain on the priming effect of nicotine on cocaine CPP. Additionally, we investigated the time lag between nicotine exposure and onset of enhancement of cocaine-induced preference. Finally, we characterized the effects of early adolescent nicotine exposure on cocaine-induced locomotor activation and sensitization in mice. It is important to examine a variety of behaviors in the same species and under the same pretreatment conditions in order to gain a comprehensive understanding of these effects. Different essay give insight to specific drug related behaviors. Acute locomotor activity use as a measure of initial sensitivity to cocaine while sensitization gives more information regarding the neuroplasticity.
3.2 Materials and methods

3.2.1 Animals

Experimentally naïve male ICR mice were obtained from Harlan Laboratories (Indianapolis, IN) and C57BL/6J and DBA2/J mice were obtained from Jackson Laboratory (Bar Harbor, ME). Adolescent mice arrived on PND 21 and weighed approximately 18-23 g at the start of the experiment, late adolescents arrived on PND 40 and weighed approximately 25-28 g at the start of the experiment, and adult mice arrived on PND 65 and weighed approximately 30-35 g. Mice were obtained from different litters and housed 4 per cage. All mice in the cage received the same treatment. They were allowed to acclimate for 7 days prior to experiments.

3.2.2 Drugs

(-)-nicotine hydrogen tartrate salt [(−)-1-methyl-2-(3-pyridyl)pyrrolidine (+)-bitartrate salt] was purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA); and cocaine HCl was provided by the Drug Supply Program of the National Institute on Drug Abuse (Rockville, MD). All drugs were dissolved in sterile saline (0.9% sodium chloride) and prepared fresh before each experiment. Nicotine was injected s.c. and cocaine was injected i.p. at a volume of 10 ml/kg body weight. Control groups received saline injections at the same volume and by the same route of administration. All doses are expressed as the free base of the drug.

3.2.3 Drug exposure protocol

Mice received nicotine during early adolescence (PND 28 to 34), late adolescence (PND 47 to 53) or adulthood (PND 70 to 76). Nicotine (0.1, 0.5 or 1 mg/kg) or saline was administered
to mice s.c. twice daily with injections approximately 6 hours apart (9:00 am and 3:00 pm) for either acute (1 day) or repeated (7 days) periods. Mice were then housed in their home cages and allowed to mature until they reached adulthood (> PND 70), at which point they were evaluated in the paradigms as described below.

3.2.4 Conditioned place preference test

Mice were tested for cocaine induced preference using the CPP test once they reached adulthood (PND>70). Essentially, the majority of the methodology in the place preference testing was the same as experiments reported in chapter 2. In the series involving DBA2/J mice the drug groups paired side assessed cocaine at a high dosage (20 mg/kg i.p.). The conditioning occurred for a longer duration (45 min). These two modifications to the methodology were utilized based on previous studies showing that DBA mice are less sensitive to cocaine (Cunningham et al. 1999).

3.2.5 Acute locomotor activity

For this experiment naïve ICR mice PND 28 and PND 70 were treated with different doses of nicotine (0.1, 0.5 or 1mg/kg s.c. twice daily) for a one day or seven-day exposure protocol. Mice were then tested for cocaine-induced hyperactivity using locomotor chambers after reaching adulthood. Cocaine dose-response curves were generated for each pretreated group (saline, 0.5 or 1 mg/kg nicotine). Mice were injected i.p with saline or various doses of cocaine (5, 10, and 15 mg/kg) and then placed into individual Omnitech Photocell Activity cages (Columbus, OH; 28 x 16.5 cm) 10 min after injection. Mice were allowed to habituate to the
chamber for 5 minutes before data collection began. Interruptions of the photocell beams (two banks of eight cells each) were then recorded for the next 30 min in 10 min intervals. Data are expressed as mean of number of photocell interruptions ± S.E.

3.2.6 Cocaine locomotor sensitization

For this study, only early adolescent mice (PND 24-30) were pretreated with saline or 0.5 mg/kg nicotine (s.c. twice daily for 7 days). After pretreatment, mice were housed until PND 70, and then a 13 day cocaine sensitization protocol began. On day 1, mice received a saline injection (i.p.) and were placed in locomotor activity chambers for a 30 minute habituation period. Mice were then removed from the chambers and activity counts were recorded. Mice were randomly divided into three groups: saline-saline, saline-cocaine, and cocaine-cocaine, which indicate the acquisition day drug and the challenge day drug, respectively. Mice were then given another injection of either saline or cocaine 20 mg/kg (i.p.), depending on the assigned group, and placed in the chambers again for a 30 minute acquisition period. This procedure was repeated on days 2-5. Days 6-12 were considered a drug free week in which the animals were not given injections or exposed to the chambers. On day 13, mice were tested again in the same manner as described for days 1-5, but mice in the saline-cocaine, and cocaine-cocaine group received a challenge dose of cocaine of 5 mg/kg (i.p.). Counts were recorded after a 30 minute test period.
3.2.7 Data analysis

For all data, graphs and statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software; San Diego, CA). Statistical analyses of all behavioral studies were performed with mixed-factor (one or two-way) ANOVA. Where appropriate, post hoc analyses (Bonferroni) were used to determine which groups were significantly (p < 0.05) different from each other.

3.3 Results

3.3.1 The influence of adolescent period of nicotine exposure on cocaine condition preference in adulthood

To address the question of whether the adolescent period during which nicotine exposure occurs influences cocaine preference in adulthood, late adolescent (PND 50) and adult (PND 70) mice were administered two doses of nicotine (0.1 or 0.5 mg/kg) for 7 days, and then tested for cocaine-induced CPP after the same drug-free period (36 days) as early adolescents (Figures 3.1 and 3.2). Once again, all mice conditioned with cocaine in the CPP test elicited a significant preference for the cocaine-paired side, as shown by one-way ANOVA (F (2, 27) =124.9; p < 0.0001). In contrast to data from early adolescent mice (Figure 2.2), mice treated with nicotine in late adolescence did not demonstrate any significant differences from mice pretreated with saline when assessed for cocaine CPP in adulthood [F (2, 27) =124.9; p > 0.05] (Figure 3.1). Similarly, mice that received either nicotine or saline during adulthood displayed approximately similar levels of preference for cocaine despite varying pretreatment [F (5, 27) =34; p > 0.05] (Figure 3.2).
Figure 3.1. The effect of late-adolescent nicotine exposure on cocaine-induced CPP in adult mice.

(A) Late adolescent mice were injected s.c. with either saline or nicotine (0.1 or 0.5 mg/kg) twice for 7 days, and were assessed for cocaine CPP test on PND 92. (B) Nicotine pretreatment during late adolescence had no effect on cocaine-induced CPP in adulthood. The x-axis represents the conditioning treatment in the CPP test. The legend represents the treatment group during late adolescence. Each bar represents the mean ± SEM of seven to eight mice. * p<0.05 from respective saline control.
Figure 3.2. The effect of adult nicotine exposure on cocaine-induced CPP after 36 days.

(A) Adult mice were injected s.c. with either saline or nicotine (0.1 or 0.5 mg/kg) two times per day for 7 days, and were assessed for the cocaine CPP test on PND 112. (B) Nicotine pretreatment during adulthood had no effect on cocaine-induced CPP in adulthood. The x-axis represents conditioning treatment in the CPP test. The legend represents the adult pretreatment group. Each bar represents the mean ± SEM of seven to eight mice. * p<0.05 from respective saline control.
3.3.2 The onset of the cocaine enhancement

Four separate groups of mice were used to determine the onset of nicotine-induced cocaine enhancement. Early adolescent (PND 28) mice were injected with either nicotine (0.5 mg/kg) or saline twice a day for 7 days, and then examined for cocaine CPP at three subsequent stages of adolescence (PND 35, 40, and 50) and in adulthood (PND 70). In Figure 3.3, two-way ANOVA (pretreatment × age of CPP) analysis showed that there was a significant effect of adolescent pretreatment \([F(1, 46)=8.66, p=0.0051]\) and onset \([F(3, 46)=5.80, p=0.0019]\) of cocaine enhancement. As shown in Figure 3.3, the onset of cocaine enhancement in the CPP test peaked around late adolescence (PND 50) and continued to adulthood (PND 70). In contrast, mice tested for cocaine-induced preference at early (PND 35) and middle (PND 40) adolescence showed no cocaine enhancement compared to their saline controls in the CPP test.
Figure 3.3. The onset of nicotine enhancement of cocaine CPP.

Early adolescent mice (PND 28) were injected s.c. with saline or nicotine (0.5 mg/kg) twice per day for 7 days, and were examined for cocaine preference in CPP test in PND (35, 40, 50, or 70). Pretreatment with 0.5 mg/kg nicotine in early adolescence significantly enhanced the cocaine preference in PND 50 and 70. The x-axis represents the date of the CPP test. The legend represents the treatment group during early adolescence. Each bar represents the mean ± SEM of seven to eight mice. *p<0.05 from respective saline-cocaine group.
3.3.3 The influence of genotype on nicotine enhancement of cocaine CPP

The effect of genotype on nicotine enhancement of cocaine CPP was investigated by treating male C57BL/6J and DBA2/J mice with nicotine during early adolescence (0.5 mg/kg twice per day for 7 days). C57BL/6J mice were conditioned with 10 mg/kg cocaine (i.p.), whereas DBA2/J mice were conditioned with 20 mg/kg cocaine. One-way ANOVA \( [F(7, 44) = 26.96, p<0.0001] \) showed that cocaine CPP was significantly enhanced in nicotine pretreated C57BL/6J mice compared to their saline pretreated controls (Figure 3.4). Interestingly, cocaine preference in nicotine pretreated DBA2/J mice did not significantly differ from their saline pretreated controls (Figure 3.5). Similar levels of nicotine were measured in the blood of C57BL/6J and DBA2/J mice treated with nicotine for 7 days (data not shown).
Figure 3.4. The effect of seven-day nicotine exposure during early-adolescent on 10 mg/kg cocaine-induced CPP in C57BL/6J mice.

(A) Early adolescent C57BL/6J mice were injected s.c. with either saline or nicotine (0.5 mg/kg) twice per day for 7 days. (B) Pretreatment with nicotine in early adolescence significantly enhanced the cocaine CPP in adult mice compared to control. The y-axis represents preference score, and the x-axis represents conditioning treatment in the CPP test. The legend represents the treatment group during early adolescence. Each bar represents the mean ± SEM of seven to eight mice. * p<0.05 from respective saline-cocaine control.
Figure 3.5. The effect of seven-day nicotine exposure during early-adolescent on 20 mg/kg cocaine-induced CPP in DBA2/J mice.

(A) Early adolescent DBA2/J mice were injected s.c. with either saline or nicotine (0.5 mg/kg) twice per day for 7 days. (B) Nicotine pretreatment during early adolescence has no effect on cocaine-induced CPP in adulthood. The y-axis represents preference score, and the x-axis represents the conditioning treatment in the CPP test. The legend represents the treatment group during early adolescence. Each bar represents the mean ± SEM of seven to eight mice.
3.3.4 Effect of adolescent nicotine exposure on cocaine-induced hyperactivity

In these studies, we examined the effects of nicotine exposure during early adolescence on the acute effects of cocaine using a locomotor activity test in adult mice. Figure 3.6 shows the results from repeated (7 days) nicotine exposure during early adolescence. Mice pretreated with nicotine (0.5 or 1 mg/kg; two times per day for 7 days) during adolescence showed a dose-related leftward shift in the cocaine dose response curve. All age groups displayed a dose-response increase in locomotor activity in response to cocaine. However, mice that were pretreated with the higher dose of nicotine (1 mg/kg) in early adolescence displayed a significant increase in cocaine-induced hyperactivity compared to those pretreated with saline [Two-way ANOVA (adolescent pretreatment x cocaine doses; $F_{(2, 56)} =10.84, p = 0.0001$]. Figure 3.7 depicts the results from acute (1 day) nicotine exposure patterns during early adolescence. No changes were observed after acute exposure compared to saline treatment (two-way ANOVA $F_{(1, 40)} =1.672, p =0.2034$]. Figures 3.8 and 3.9 shows the results from studies where pretreatment occurred in adulthood. As anticipated, no significant differences were seen based on pretreatment injections, confirming that the effect seen in Figure 3.6 was unique to the early adolescent period.
Figure 3.6. The effect of repeated nicotine exposure during early-adolescence on cocaine-induced hyperactivity in adult mice.

(A) Early adolescent mice (PND 28) were injected s.c. with saline or various doses of nicotine (0.5 or 1 mg/kg) twice daily for 7 days, and were examined for cocaine hyperactivity on PND 70. (B) Pretreatment with 1 mg/kg of nicotine in early adolescence significantly enhanced cocaine locomotor activity in adult mice, while pretreatment with 0.5 mg/kg failed to enhance cocaine locomotor activity during the 30-minute test period. Data are expressed as the number of photocell interruptions ± SEM for 6 mice per group. The legend represents the treatment group during early adolescence. * p<0.05 from respective saline-cocaine control.
Figure 3.7. The effect of acute nicotine exposure during early-adolescence on cocaine-induced hyperactivity in adult mice.

(A) Early adolescent mice (PND 28) were injected s.c. with saline or nicotine (0.5 mg/kg) twice daily for 1 day, and were examined for cocaine hyperactivity on PND 70. (B) Pretreatment with 0.5 mg/kg nicotine failed to enhance cocaine during the 30-minute test period. Data are expressed as the number of photocell interruptions ± SEM for 6 mice per group. The legend represents the treatment group during early adolescence.
Figure 3.8. The effect of repeated nicotine exposure in adult mice on cocaine-induced hyperactivity later in life.

(A) Adult mice (PND 70) were injected s.c. with saline or various doses of nicotine (0.5 or 1 mg/kg) twice daily for 7 days, and were examined for cocaine hyperactivity on PND 112. (B) Pretreatment with nicotine (0.5 or 1 mg/kg) in adulthood failed to enhance cocaine locomotor activity during the 30-minute test period. Data are expressed as the number of photocell interruptions ± SEM for 6 mice per group. The legend represents the treatment group during early adolescence.
Figure 3.9. The effect of acute nicotine exposure in adult mice on cocaine-induced hyperactivity later in life.

(A) Adult mice (PND 70) were injected s.c. with saline or nicotine (0.5 mg/kg) twice daily for 1 day, and were examined for cocaine hyperactivity on PND 112. (B) Pretreatment with nicotine (0.5 mg/kg) in adulthood failed to enhance cocaine locomotor activity during the 30-minute test period. Data are expressed as the number of photocell interruptions ± SEM for 6 mice per group. The legend represents the treatment group during early adolescence.
3.3.5 Effect of adolescent nicotine exposure on locomotor sensitization to cocaine

We then examined the effects of early adolescent nicotine treatment on cocaine-induced behavioral sensitization. Figure 3.10 shows data from mice that were pretreated with 0.5 mg/kg nicotine for 7 days in early adolescence (solid bars) compared to data from saline-pretreated mice (non-solid bars). During the acquisition period, mice that were treated with cocaine (20 mg/kg) demonstrated enhanced locomotor activity as expected, with no differences due to adolescent nicotine pretreatment (p<0.05 compared to saline-saline). On the challenge day, two groups received an injection of cocaine i.p. (5 mg/kg). Both saline- and nicotine-pretreated mice that were treated with cocaine during the acquisition period displayed enhanced locomotor activity compared to the mice treated with saline during acquisition. However, mice that were pretreated with nicotine in adolescence showed a significant increase in cocaine-induced locomotor activity compared to saline-pretreated animals. These results demonstrate that early adolescent nicotine exposure enhances the induction of locomotor sensitization to cocaine.
Figure 3.10. Effect of early adolescent nicotine exposure on behavioral sensitization to cocaine.

Early adolescent mice were pretreated with either saline (non-solid bars) or 0.5 mg/kg nicotine (solid bars) for 7 days, and were tested for cocaine-induced locomotor sensitization in adulthood. Treatment groups are represented by acquisition drug-challenge drug in the legend (ex. sal-coc = saline during acquisition and cocaine on challenge day) *p<0.05 from sal-sal control on the same day; # p<0.05 from sal-coc group; $p<0.05 from saline pretreated coc-coc group.
3.4. Summary

Our data revealed that a 7 days exposure to low doses of nicotine in adolescent mice enhanced several behavioral effects of cocaine in adulthood: CPP, locomotor activation and sensitization. Our results also clearly show that the age at which the animals are exposed to nicotine have a great impact. Nicotine exposure in early (but not late) adolescence or in adulthood enhanced cocaine’s preference in the CPP test. The enhancement of cocaine behavioral responses depended upon the age and length of nicotine exposure. These results suggest that early adolescence is a critical period for behavioral plasticity induced by nicotine. Utilizing the same repeated nicotine treatment regimen used in ICR mice, our results showed that the enhancement of cocaine reward was replicated in C57 but not DBA mice. This strain difference suggests the possible involvement of genetic factors in the effects of nicotine in adolescence, and could help in understanding individual differences in cocaine-taking behavior, and the drug abuse liability observed in humans.
CHAPTER FOUR

Effects of early-adolescent nicotine exposure on altering the reward-related effects and dependence with other drugs of abuse in adult mice

4.1 Introduction

Adolescence is often the time for novelty seeking and risk-taking behaviors during which high levels of drug experimentation are reported. Epidemiological studies have demonstrated that adolescent tobacco smokers are more likely to proceed and abuse other drugs as adults (Kandel et al., 1992). Similarly, adolescent nicotine exposure in rodents has been shown to affect cocaine and alcohol preferences and sensitivities (Collins & Izenwasser, 2004; Kelley & Middaugh, 1999; Kelley & Rowan, 2004). Our results in Chapters Two and Three demonstrated that repeated nicotine exposure during early adolescence yields persistent changes in adult mice’s behavioral responses to cocaine. Given that many abused drugs share the same molecular “circuits,” we can speculate that early adolescence exposure to nicotine may lead to persistent changes in sensitivity to other abused drugs as well.

In this chapter, follow-up studies were undertaken to determine if this enhancement seen with cocaine generally applies to other abused drugs. We hypothesized that exposure to nicotine during early adolescence would cause persistent changes in the reward-related effects of abused drugs in general. Using the same experimental design as in Chapter Three, we examined the effects of adolescent nicotine on different abused drugs that have different initial cellular targets. In particular, we studied the effects of nicotine exposure on morphine and amphetamine abuse in the conditioned place preference test. Moreover, we investigated if cocaine exposure during
adolescence had an effect on nicotine reward in adult mice. Having already examined the adolescent nicotine effect on the mice’s reward response to cocaine and morphine, we exposed the mice to nicotine during early adolescence and tested for morphine physical dependence once the animals reached adulthood.

4.2 Materials and methods

4.2.1 Drugs

(-)-nicotine hydrogen tartrate salt [(-)-1-methyl-2-(3-pyridyl) pyrrolidine (+)-bitartrate salt] was purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA); amphetamine, cocaine, morphine, and naloxone were supplied by the National Institute on Drug Abuse (Rockville, MD). All drugs were dissolved in sterile saline (0.9% sodium chloride) and prepared fresh before each experiment. Amphetamine, morphine, and nicotine were injected s.c. while cocaine and naloxone were i.p. at a volume of 10 ml/kg body weight. Control groups received saline injections at the same volume and by the same route of administration. All doses are expressed as the free base of the drug.

4.2.2 Drug exposure protocol

Similar to the previous experiments reported in Chapter Three, naïve ICR mice received nicotine (cocaine in one experiment) during early adolescence (PND 28 to 34) or adulthood (PND 70 to 76). Nicotine (0.5 mg/kg), cocaine (10 or 20 mg/kg) or saline was administered to mice s.c. twice daily with injections approximately 6 hours apart (9:00 am and 3:00 pm) for 7 days. Mice were then housed in their home cages and allowed to mature until they reached
adulthood (> PND 70), at which point they were evaluated in the CPP test or withdrawal paradigms as described below.

4.2.3 Conditioned place preference test

Mice pretreated with nicotine were tested for amphetamine or morphine preference, while those treated with cocaine were tested for nicotine preference using the CPP test after they reached adulthood. The CPP protocol utilized in Chapter two was implemented here, except the nicotine pretreated drug groups received morphine (2.5, 5.0, or 10.0 mg/kg s.c.) or amphetamine (0.2, 5.0, or 10.0 mg/kg s.c.), while the cocaine pretreated group received nicotine (0.1 or 0.5 mg/kg s.c.) in one side and saline in the opposite side.

4.2.4 Induction of morphine physical dependence

Another set of early adolescent (PND 28) mice was treated with saline or nicotine (0.5 mg/kg; s.c. twice daily for seven days). When the mice reached adulthood (PND 70), they were randomly divided into four groups based on their adolescent pretreatment (saline or nicotine) and the following adulthood and challenge treatments (saline and saline or morphine and naloxone). On day one, depending on the groups’ indicated adulthood treatment, the mice were injected with saline or 50 mg/kg of morphine s.c. three time daily at 9:00 a.m., 12:00 p.m., and 3:00 p.m. On day two, the morphine groups received 75 mg/kg of s.c. three times per day. On day three, the morphine dose was increased to 100 mg/kg thrice daily. On day four, 100 mg/kg of morphine was injected once at 9:00 a.m. Two hours later, the mice were injected i.p. with saline or naloxone (2.0 mg/kg). Immediately thereafter, the animals were placed individually in separate glass beakers and observed for typical morphine withdrawal signs that included head shakes,
paw tremors, body tremors, and backing, ptosis, curls, and jumps. Results were expressed as the mean ± S.E.M. number of signs displayed by mice during the 30-minute observation period.

4.3 Results

4.3.1 Effects of early-adolescent nicotine exposure on morphine-induced conditioned place preference

To assess the effects of early-adolescent nicotine exposure on morphine CPP in adulthood, the mice were pretreated with nicotine (0.5 mg/kg s.c.; two injections daily for seven days) during early adolescence (PND 28–34) and then they were allowed to mature to adulthood (PND 70) before CPP testing (Figure 4.1). A two-way ANOVA (adolescent treatment × CPP treatment) with Bonferroni Posthoc test showed significant effects of adolescent treatment \[F(1, 46) = 13.31, p = 0.0007\] and CPP treatment \[F(3, 46) = 18.08, p < 0.0001\], with no significant interaction between adolescent treatment × CPP treatment \[F(3, 46) = 0.5690, p = 0.6383\]. The differences in the morphine dose-response in addition to the dose-response curves for the two groups were graphed (Figure 4.2). Interestingly, the pretreatment with nicotine at 0.5 mg/kg during adolescence produced a leftward shift in the morphine-dose-response curve, and subsequent doses of 5 and 10 mg/kg of morphine evoked a significant CPP response in adulthood compared to saline controls. These results show that early-adolescent nicotine exposure increases the CPP scores of morphine in adult mice.
Figure 4.1. The effect of early-adolescent nicotine exposure on morphine CPP in adult male mice.

(A) As shown in the upper panel, early-adolescent mice were injected s.c. with saline or nicotine (0.5) twice daily for seven days and were conditioned with differing doses of morphine (2.5, 5.0, or 10.0 mg/kg) in PND 70. (B) The legend represents the treatment group during early adolescence. Each bar represents the mean ± SEM of seven to eight mice. *p<.05 from respective saline control. ^ p<.05 from respective saline-morphine control.
Figure 4.2. The effect of early-adolescent nicotine exposure on morphine dose-response curve for CPP in adult male mice.

Early-adolescent mice were injected s.c. with saline or nicotine (0.5) twice daily for 7 days and were conditioned with differing doses of morphine (2.5, 5.0, or 10.0 mg/kg) in PND 70. Repeated nicotine exposure in early adolescence produced a leftward shift in the morphine dose response curve. The y-axis represents preference score and the x-axis expresses the conditioning treatment in the CPP test. The legend represents the treatment group during early adolescent. Each point represents the mean ± SEM of seven to eight mice. *p<.05 from respective saline-morphine control.
4.3.2 Effects of adolescent nicotine exposure on morphine withdrawal in adult mice

The mice were pretreated with nicotine (0.5 mg/kg s.c.; two injections each day for seven days) during early adolescence (PND 28–34) and then they were allowed to mature to adulthood (PND 70) before morphine withdrawal testing. A two-way ANOVA (adolescent treatment × adult treatment) with a Bonferroni Posthoc test indicated that naloxone administration in morphine-treated mice significantly increased the expression of somatic withdrawal signs \( [F_{(1, 20)} = 63.11, p < 0.0001] \). However, there were no significant effects of adolescent nicotine treatment \( [F_{(1, 20)} = 0.0006, p = 0.9806] \) (Figure 4.3). In addition, a separate analysis of individual signs revealed no significant effect of adolescent pretreatment.
Figure 4.3 The effect of early-adolescent nicotine exposure on morphine withdrawal in adult male mice.

(A) As shown in the upper panel, early-adolescent mice were injected s.c. with nicotine (0.5mg/kg twice per day for seven days) and then at PND 70 tested for morphine somatic signs withdrawal following naloxone administration. (B) The y-axis represents number of signs displayed by mice during the 30-minute observation period. Each bar represents the mean ± SED of six mice. *p<0.05 from the respective saline group.
4.3.3 Effect of early-adolescent nicotine exposure on amphetamine-induced conditioned place preference in adult mice

Early-adolescent mice (PND 28–34) were pretreated with nicotine (0.5 mg/kg twice a day for seven days), and at PND 70 they were conditioned to amphetamine (0.2 mg/kg). Figure 4.4 shows that all the mice conditioned with amphetamine in the CPP test developed a significant preference for the drug-paired side as compared to their respective saline controls. Interestingly, compared to mice that received the saline pretreatment, the mice that were pretreated with nicotine during adolescence displayed a significantly enhanced level of amphetamine CPP \[F(3, 26) = 17.18, p<0.05\] at 0.2 mg/kg in adult mice. It is possible that mice reach a ceiling effect at a dose of 5 mg/kg of amphetamine and that why we could not see any further enhancement as a result of the adolescent pretreatment.
Figure 4.4. Effects of early-adolescent nicotine exposure on amphetamine-induced CPP in adulthood.

(A) As shown in the upper panel, early adolescent mice were injected s.c. with saline or nicotine (0.5 mg/kg) twice daily for seven days and were conditioned with different doses of amphetamine (0.2, 5.0, or 10.0 mg/kg) in PND 70. (B) The x-axis expresses the conditioning treatment in the CPP test. The legend represents the treatment group during early adolescence. Each bar represents the mean ± SEM of seven to eight mice. *p<.05 from respective saline-amphetamine control.
4.3.4 The impact of the sequential order between nicotine and cocaine on drug-induced CPP

Our previous results established that repeated nicotine exposure during early adolescence enhanced the cocaine, morphine, and amphetamine place preference in CPP test. It was important for us to examine if the nicotine effect is bidirectional or not. To investigate this, early-adolescent mice (PND 28) were pretreated with cocaine (10 or 20 mg/kg, two injections each day for a total of seven days), and at PND 70 they were conditioned to nicotine (0.1 or 0.5 mg/kg). Cocaine pretreatment did not enhance nicotine CPP in adult mice. Intriguingly, mice pretreated with cocaine (10 mg/kg, twice daily for a week) in early adolescence showed condition place aversion to nicotine (0.5 mg/kg) compared to saline-treated mice (Figure 4.5).
Figure 4.5. The effect of early-adolescent cocaine exposure on nicotine-induced CPP in adulthood.

(A) As shown in the upper panel, early adolescent mice were injected i.p with saline or cocaine (10 or 20 mg/kg) twice daily for 7 days and were conditioned with nicotine (0.5 mg/kg) in PND70. (B) The x-axis expresses adolescent treatment in the CPP test. The legend represents the treatment group during early adolescent. Each bar represents the mean ± SEM of seven to eight mice. ^p<.05 from respective saline control. *p<.05 from saline-nicotine.
4.4. Summary

Our results demonstrated that the enhancement of cocaine reward is replicated with other abused drugs when utilizing a nicotine treatment regimen during early adolescence, with significant enhancement of morphine (Figure 4.1) and amphetamine (Figure 4.4) preferences in the CPP test for adult mice. Similarly, a study done by Kota et al. (2009) showed that early adolescent nicotine exposure significantly enhanced the nicotine reward later in life. Furthermore, this exposure regimen caused a leftward shift of the morphine dose-response curve in adult mice. In contrast, an adolescent nicotine pre-treatment did not affect the somatic sign in the morphine withdrawal test. Finally, there was no bidirectional effect between cocaine and nicotine.
CHAPTER FIVE

Mechanisms underlying adolescent nicotine-enhanced drug reward in adult mice

5.1 Introduction

Cocaine and amphetamine exposure during adolescence induced an increase in the magnitude of ∆FosB, a stable transcription factor, in NAc of mice when compared to mice exposed in adulthood (Ehrlich et al., 2002). In addition, over-expression of the transcription factor ∆FosB in the brain enhanced sensitivity to cocaine and morphine behavioral effects (Nestle et al., 2008). ∆FosB persists in neurons for long periods of time and is believed to contribute to increased susceptibility to the long-term behavioral effects of addictive drugs and increased drug-seeking behaviors (Nestle et al., 2008). ∆FosB is one of the molecular adaptations that could explain the protracted behavioral response reported in Chapters Two through Four.

The primary goal of this chapter was to identify specific alterations in ∆FosB expression in NAc and PFC that occur subsequent to early nicotine exposure. ∆FosB levels were measured one week after the beginning of nicotine exposure, which was also the last day of the exposure. ∆FosB levels were measured again 4 weeks after the beginning of nicotine exposure, and again after the cocaine CPP test, which was 6 weeks after the beginning of exposure. These measurements were done for mice exposed during adolescence (exposure beginning PND 28) and during adulthood (exposure beginning PND 70).

Also, we investigated the involvement of nicotinic receptors in mediating the sensitization of cocaine’s behavioral effects by using antagonists with varying subtype
selectivity. Results from these experiments will help elucidate part of the mechanisms involved in the nicotine priming effect on cocaine behavioral effects in adulthood.

5.2 Materials and methods

5.2.1 Animals

Experimentally naïve male ICR mice were obtained from Harlan Laboratories (Indianapolis, IN). Adolescent mice arrived on PND 21 and weighed approximately 18–23 grams at the start of the experiment, and adult mice arrived on PND 65 and weighed approximately 30–35 grams. Mice were obtained from different litters and were housed 4 per cage. They were allowed to acclimate for 7 days prior to experiments.

5.2.2 Drugs

(−)-nicotine hydrogen tartrate salt [(−)-1-methyl-2-(3-pyridyl)pyrrolidine (+)-bitartrate salt], mecamylamine hydrochloride [2-(methylamino) isocamphane hydrochloride], methyllycaconitine (MLA), and dihydro-β-erythroidine (DHβE) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA); cocaine HCl was provided by the Drug Supply Program of the National Institute on Drug Abuse (Rockville, MD). All drugs were dissolved in sterile saline (0.9% sodium chloride) and prepared fresh before each experiment. All compounds were injected subcutaneously except for the cocaine, which was injected intraperitoneally at a volume of 10 ml/kg body weight. Control groups received saline injections at the same volume and by the same route of administration. All doses are expressed as the free base of the drug.
5.2.3. Conditioned place preference test

Mice were tested for cocaine-induced preference using the CPP test once they reached adulthood. An unbiased CPP test was utilized in this study, as described in Chapter Two.

5.2.4. Western blotting

Twenty-four hours after their last treatment, mouse brains were harvested via rapid decapitation and sectioned with razor blades in a stainless steel brain block chilled on ice. Coronal slices were obtained, and core punches were taken of NAc and PFC (which was haphazardly dissected to include medial prefrontal cortex as well as cingulate, motor, and somatosensory cortices). Dry ice was used to rapidly freeze tissue samples until it was time to process them for Western blot procedures. Immunoblotting was performed as previously described (Sim-Selley et al., 2006; Zachariou et al., 2006). Tissue was homogenized in 20 mM HEPES buffer (pH 7.8) with 0.4 M NaCl, 20.0% glycerol, 5.0 mM MgCl2, 0.5 mM EDTA, 0.1 mM EGTA, and 1% NP-40 (EMSA buffer) containing 500 µM dithiothreitol and Halt™ protease inhibitor cocktail. Samples were loaded in 10% Tris-HCl gels and separated by electrophoresis. Gels were transferred onto nitrocellulose paper, blocked in 0.1 M TBS with 5% Carnation™ instant nonfat dry milk for 1 hour, incubated in FosB (1:1000; Cell Signaling Technology, Beverly, MA, USA) and α-tubulin (1:5000; Upstate, Temecula, CA, USA) antibodies in 0.1 M TBS containing 0.1% Tween-20 (TBST) with 5% nonfat dry milk. Blots were washed 3 x 10 minutes in TBST and incubated with Alexa 680 goat anti-rabbit IgG (1:12000) and Alexa 800 goat anti-mouse IgG (1:12000) in TBST for 45 minutes at room temperature. Fluorescent intensity was visualized using the Odyssey LI-COR infrared scanner. LI-COR software version 2.1 was used to measure integrated
intensity between treatments for the band of interest, with subtraction of the background (average of intensities 3 border widths above and below the band).

5.3. Results

5.3.1. Nicotine receptor subtypes underlying early-adolescent nicotine enhancement of cocaine CPP

The role of different nicotine receptor subtypes in enhancing the effects of cocaine was investigated using antagonists with varying subtype selectivities. Studies were first conducted by pretreating adolescent mice with nicotine plus the nonselective nicotinic antagonist mecamylamine to determine whether blocking nicotinic receptors prevents nicotine-induced cocaine enhancement in adulthood. Figure 5.1 shows that pretreatment of early adolescent mice with mecamylamine (2mg/kg s.c.) and nicotine preventedblocked enhancement of cocaine CPP in adulthood \[F(4, 28) = 4.169, p = 0.009\]. These results suggest that the activation of nAChRs is required for nicotine-induced enhancement of cocaine in the CPP test. Furthermore, DHβE (2 mg/kg) and MLA (8 mg/kg) antagonists for β2* and α7 nAChRs subtypes, respectively, administered before daily nicotine pretreatment in adolescence, also blocked enhanced cocaine CPP in adulthood (Figure 5.1). Pretreatment with nicotinic antagonists alone during early adolescence did not enhance cocaine CPP in adulthood (Table 5.1).
Figure 5.1. Blockade of nicotine priming effects on cocaine CPP by various nicotinic antagonists.

(A) As shown in the upper panel, early-adolescent mice (PND28) were pretreated s.c. with mecamylamine hydrochloride (MEC), methyllycaconitine (MLA), or dihydro-β-erythroidine (DHβE) before daily nicotine pretreatment twice per day for 7 days. (B) The legend represents the treatment group during early adolescence. Each bar represents the mean ± SEM of 6 to 8 mice. * p < .05 from respective saline control; ^ p < .05 from respective saline-cocaine.
<table>
<thead>
<tr>
<th>Early-adolescent treatment</th>
<th>CPP treatment</th>
<th>Preference score</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEC-SAL</td>
<td>Cocaine</td>
<td>162.11</td>
</tr>
<tr>
<td>DHβE-SAL</td>
<td>Cocaine</td>
<td>154.06</td>
</tr>
<tr>
<td>MAL-SAL</td>
<td>Cocaine</td>
<td>176.35</td>
</tr>
</tbody>
</table>

**Table 5.1**

Effect of nicotinic antagonists pretreatment during early-adolescent on cocaine CPP mecamylamine hydrochloride (MEC), methyllycaconitine (MLA), dihydro-β-erythroidine (DHβE) and saline (SAL)
5.3.2 ΔFosB level in NAc after repeated nicotine exposure in early-adolescent and adult mice

Results from Chapters Two and Four establish that repeated nicotine exposure during early adolescence enhanced cocaine, morphine, and amphetamine placement preference in CPP tests. To understand the mechanisms involved in our protracted behavioral response, we first examined changes in ΔFosB activity after chronic nicotine exposure.

Early-adolescent mice (PND 28) were pretreated with saline or nicotine (0.5 mg/kg, 2 injections each day for a total of 7 days), and on day 8 (PND 35) NAc was dissected and prepared for Western blot analysis. Results show that chronic nicotine use induces a significant increase (approximately four fold) in ΔFosB expression \([p < 0.005]\) compared to saline controls (Figure 5.2). Similarly, adult mice (PND 70) received the same pretreatment regimen, and NAc was dissected 24 hours after the last treatment (PND 77) and prepared for Western blot analysis. Results showed that chronic nicotine induces an increase in ΔFosB expression compared to saline controls (Figure 5.2). However, the increase in ΔFosB expression was not significant \([p > 0.05]\) for mice exposed in adulthood to the same dose of nicotine. Further analysis showed that the difference in ΔFosB expression between adolescent and adult mice exposed to nicotine was significant as well \([p < 0.005]\).
Figure 5.2. ΔFosB level in NAc after 24 hours’ abstinence from nicotine exposure.

Western blot analysis revealed a significant effect of early-adolescent nicotine pretreatment on levels of ΔFosB in NAc compared to mice pretreated with nicotine at PND > 70. Results were represented as protein levels over α-tubulin expressed as a percentage of saline control (4 animals per group): *p < 0.05 from respective saline and ^ p < 0.05 from adolescents also treated with nicotine.
Furthermore, mice were examined 36 days after their last treatment. Figure 5.3 shows that ΔFosB levels declined from the initial testing period; however, levels in mice pre-exposed to nicotine in adolescence were still significantly higher than levels in mice exposed to saline in adolescence or adulthood and in mice exposed to nicotine in adulthood [p < 0.05].
Figure 5.3. ΔFosB level in NAc after 35 days’ abstinence from nicotine exposure.

(Early adolescent nicotine pretreatment has a significant effect on levels of ΔFosB in NAc compared to the respective saline control adult-nicotine group. Results were represented as protein levels over α-tubulin expressed as a percentage of saline control (4 animals per group): *p < 0.05 from respective saline and ^ p < 0.05 from adolescents also treated with nicotine.)
Figure 5.4 illustrates the data collected when ΔFosB levels were measured after cocaine treatment during CPP testing. Cocaine treatment during CPP tests significantly increased ΔFosB levels in all groups compared to saline treatment in adult mice \[ F (3, 15) = 11.16, p < 0.05 \]. Furthermore, mice exposed to nicotine during adolescence showed a significant increase in ΔFosB levels after cocaine treatment in CPP testing compared to mice exposed to nicotine in adulthood. These results suggest an age-dependent effect of nicotine on ΔFosB expression \[ p < 0.05 \]. Pretreatment with nicotine during adulthood has no significant effect on ΔFosB expression after cocaine CPP when compared to the saline-cocaine control group \[ p > 0.05 \].
Figure 5.4. ΔFosB level in NAc 24 hours after last cocaine exposure during CPP.

Cocaine significantly enhanced ΔFosB levels in mice pretreated with nicotine during adolescence compared to saline-treated mice and mice pretreated with nicotine in adulthood, and also significantly enhanced ΔFosB levels in mice conditioned with cocaine, compared to mice conditioned with saline. Results are represented as protein levels over α-tubulin expressed as a percentage of saline control (4 animals per group): ^ p < 0.05 from respective saline * and p < 0.05 from respective adult-nicotine-cocaine and adult-saline-cocaine.
5.3.2. ∆FosB level in PFC after repeated nicotine exposure in early adolescent and adult mice

∆FosB levels in PFC were also investigated. Early-adolescent and adult mice received nicotine (from PND 28–34 and PND 70–76, respectively); 24 hours after last treatment, PFC was dissected and prepared for Western blot analysis. Figure 5.5 shows that nicotine pretreatment during early adolescence increased levels of ∆FosB, yet there was no significant effect compared to mice exposed to nicotine in adulthood or to mice exposed to saline. Mice exposed to nicotine in adulthood also had higher levels of ∆FosB compared to control mice exposed to saline; however, the increase was not significant.

∆FosB levels were measured after CPP tests on these mice. The results, displayed in Figure 5.6, showed that cocaine has a tendency to increase ∆FosB expression compared to saline treatment in CPP tests. However, there was no difference in the effect of nicotine pretreatment in adulthood or adolescence on ∆FosB expression after CPP testing.
Figure 5.5. ΔFosB level in PFC after 24 hours’ abstinence from nicotine exposure.

Nicotine pretreatment had no significant effect on levels of ΔFosB in PFC compared to mice pretreated with saline or nicotine during adulthood. Results are represented as protein levels over α-tubulin, expressed as a percentage of saline control (4 animals per group).
Figure 5.6. ΔFosB level in PFC 24 hours after last cocaine exposure during CPP.

Western blot analysis revealed no significant effect of nicotine pretreatment on levels of ΔFosB in PFC compared to mice pretreated with saline. Results are represented as protein levels over α-tubulin, expressed as a percentage of saline control (4 animals per group).
5.4. Summary

In the present study, we report that enhancement of cocaine reward in mice pretreated with nicotine during early adolescence is mediated by neuronal nicotine receptors (mainly β2* and α7). Excitingly, data of ΔFosB experiments revealed significant effects of age and nicotine pretreatment in NAc. Indeed, nicotine pretreatment was able to increase the ΔFosB levels in NAc significantly in early adolescence compared to adult mice. This increase in the ΔFosB level has persisted for several weeks; however, no significant effect of pretreatment or age was found in PFC. These differential neuroadaptations may explain why nicotine use during early adolescence may carry a greater risk than nicotine use during adulthood.
CHAPTER SIX

Discussion

6.1. Rationale and summary of overall hypothesis

Nicotine is one of the most commonly used drugs by the adolescent population. Adolescent tobacco dependency is a complex disease. Social, environmental, and genetic factors, along with a biological basis, all play significant roles in the initiation of tobacco use and contribute to the progression from use to addiction and/or abuse. Adolescent tobacco use still represents a major challenge to the future of public health. In 2011, an estimated 68.2 million Americans aged 12 and older were current (within the past 30 days) users of tobacco products (SAMHSA, 2011). Unfortunately, the addictive property of nicotine is an obstacle for those with a strong desire to quit. Only about 3–5% of those who use and/or are addicted to tobacco and eventually attempt to quit remain abstinent 6–12 months later (Hughes et al., 2004). Adolescence is the final developmental period leading to adulthood, occurring between ages 12 to 18 years in humans and between 28 to 60 postnatal days in rodents (Spear, 2000; Laviola et al., 2003). During this critical period, the brain is undergoing major developmental changes in addition to various biological, hormonal, behavioral, and other changes (for review Spear, 2000). These changes may contribute to individuals’ participation in activities such as risk taking and novelty seeking, increased social interactions, and increased vulnerability to abuse of nicotine and other drugs of abuse.

The majority of the literature links tobacco use in human adolescence and subsequent developmental problems in adulthood, including effects on later tobacco, alcohol, cocaine, and
other illicit drug use and mental health (Anthony & Petronis, 1995; Clark et al., 1998; Kandel et al., 1992). Compared with nonsmokers, early smokers at Grade 7 were more likely to experience severe types of substance abuse (binge drinking and hard drug use) later in life. Furthermore, teens with histories of cigarette use by age 16 were over 9 times more likely to begin using heroin by age 32 (Johnson et al., 1995). Indeed, early onset of drug abuse has been hypothesized to increase the risk of later drug addiction (Anthony & Petronis, 1995; Clark et al., 1998; Kandel et al., 1992). Given the propensity for drug use later in life, it is vital to understand the behavioral and neurochemical processes that occur when adolescents are exposed to nicotine.

Exposure to nicotine may have distinctive effects during adolescence, making this a more vulnerable period for long-term effects than adulthood and contributing to an increased risk of developing drug dependence in adulthood (for review Slotkin, 2002). Indeed, studies in rodents have demonstrated heightened rewarding and reinforcing effects of nicotine (Adriani et al., 2002; Belluzzi et al., 2004; Kota et al., 2007; Vastola et al., 2002) and attenuated affective withdrawal signs of nicotine (Kota et al., 2007; O’Dell et al., 2006). This valuable information suggests that there are major differences between the mechanisms that drive nicotine use in adolescents and in adults.

Despite the important contribution of understanding the impact of adolescent smoking on drug-dependence behavioral outcomes in adulthood, few studies assess the effect of nicotine exposure during adolescent on drugs of abused behavioral response occurring in adulthood. Additionally, the studies addressing the mechanisms underlying nicotine priming effects are lacking; the aim of this study was therefore to focus on the impact of adolescent nicotine exposure on the subsequent behavioral response toward cocaine, amphetamine, and morphine. While it is difficult to explore and understand the causes for this age-related vulnerability in
human subjects, the use of animal models allows the exploration of the various behavioral and molecular mechanisms involved.

We hypothesized that adolescents exposed to low doses of nicotine would demonstrate increased sensitivity to typically abused drugs when compared to adults who were first exposed to nicotine in adulthood. To this end, our studies have focused on establishing and characterizing a mouse model to study the effects of nicotine in adolescence. Secondly, our studies concentrated on identifying the role of nAChR subtypes in nicotine priming effects on cocaine use in adults. Finally, we conducted biochemical studies to partially elucidate the mechanism underlying the relationship between adolescent nicotine exposure and enhanced behavioral responses to cocaine and other drugs of abuse in adult mice. We hypothesized that these changes in cocaine behavioral responses were a result of nicotine-induced, brain region–specific changes in ΔFosB expression.

6.2. Early adolescence presents a unique period of vulnerability to nicotine effects

In the current studies, we have shown that a 7-day treatment with a dose of 0.5 mg/kg nicotine during early adolescence produces unique changes in the response to drugs of abuse in adulthood, particularly cocaine. We showed in chapter two and three that nicotine administration during early adolescence enhanced cocaine CPP preference, acute locomotor activity, and sensitization to cocaine in adult mice in a dose-related manner. These behavioral changes are long-lasting and dependent upon the age at which nicotine pretreatment is administered. Adolescent mice exposed to low doses of nicotine after repeated, but not acute, administration displayed enhanced behavioral responses (e.g., CPP and locomotor activation) to cocaine as adults, suggesting the involvement of plastic events after exposure to nicotine. However, repeated pretreatment with nicotine in early adolescence increased cocaine-induced
CPP in a dose-related manner, increased tendency to enhance cocaine CPP after pretreatment with a dose of 0.1 mg/kg nicotine s.c., and significantly enhanced cocaine preference after pretreatment with 0.5mg/kg nicotine s.c.; however, doubling the nicotine dose to 1mg/kg lost the effect. Thus, low doses of nicotine appear to selectively influence processes relating to cocaine-induced behavioral plasticity after exposure to the drug in adolescence. At a low dose of 0.5 mg/kg, nicotine pretreatment shifted the cocaine dose–response curve to the left. Additionally, the effects of nicotine on sensitization to cocaine depend on the age of exposure to the drug. Indeed, an increased behavioral response to cocaine was observed when nicotine exposure occurred during early adolescence (PND 28–34), but not during late adolescence or adulthood. These findings indicate that early adolescence is a crucial period of vulnerability to the effects of nicotine. In the same manner, Belluzzi et al. (2004) demonstrated that early-adolescent (PND 28) animals displayed a preference for an environment paired with a single injection of nicotine, whereas late-adolescent (PND 38) and adult animals did not display this effect.

Our results are consistent with previous behavioral studies that have shown differences in responses to cocaine in rodents treated with nicotine during adolescence compared with animals that receive nicotine as adults. In both adolescent (Collins & Izenwasser 2004; Dao et al., 2011; McQuown et al., 2007) and adult rats (McMillen et al., 2005) pre-exposure to nicotine during adolescence sensitized to cocaine-mediated locomotor responses and CPP and increased the self-administration of cocaine. In contrast to these findings, Kelley and Rowan (2004) found that C57BL/6J mice demonstrated a decrease in a cocaine-induced preference as measured by CPP after adolescent nicotine exposure. This difference could be due to nicotine dose (3 mg/kg vs. 0.5 mg/kg), the length of nicotine exposure (25 days vs. 7 days) used in the Kelley and Rowan study, or the age of animals during CPP testing (PND 142 vs. PND 72). As the results from the
experiment with the mini-pump exposure agreed with the findings of Kelly and Rowan (2004), it seems that the duration of the exposure to nicotine during adolescence has a significant effect on the cocaine behavioral response. An exposure lasting 7 days resulted in an enhanced cocaine behavioral response, while an exposure lasting the whole adolescent period decreased cocaine behavioral response. Furthermore, researchers noted that this exposure led to an increase in cocaine’s motor-activating effects, in agreement with the results from our acute locomotor study of cocaine (Chapter Three).

In contrast to our findings, McQuown et al. (2007) reported that exposure to nicotine for 4 days during early adolescence enhanced the reinforcing effects of cocaine in rat i.v. self-administration, evident one day after cessation of the drug. In our study, however, CPP immediately following adolescent treatment did not result in enhanced CPP. The enhancement in CPP in nicotine-pretreated mice (PND 28) started to peak around mid-adolescence (PND 50). The discrepancy between the studies could be attributed to differences in exposure regimen (4-day i.v. vs. 7-day s.c.), species (rat vs. mouse), and cocaine behavioral effects measured (CPP vs. self-administration).

In addition, our data suggest the importance of nicotine delivery on human behavior. Continuous delivery of nicotine during adolescence failed to enhance cocaine response in adult mice, perhaps suggesting that the length of time during which receptors are exposed to nicotine concentrations is crucial. Because nicotine enters the body all at once, as opposed to over an extended period of time, intermittent nicotine administration is necessary to cause enhanced cocaine response in adulthood. This finding is important because this method of delivery mimics nicotine exposure in human adolescents and adults. It is also possible that this difference in cocaine CPP may be attributable in part to differences in the total daily dose of nicotine.
administered by each method; our results show that the levels of nicotine in the blood for the subcutaneous injection and drinking water methods were similar and lower than levels for the mini-pump method.

Similar to CPP results, the exposure of early-adolescent mice to nicotine also enhanced cocaine acute locomotor hyperactivity and locomotor sensitization in adulthood in a dose-dependent manner. The enhancement of cocaine acute locomotor activity was seen at doses of 0.5 mg/kg of nicotine and 1 mg/kg nicotine. Yet only pre-exposure to 1 mg/kg of nicotine was statistically significant, which was greater than the dose (0.5 mg/kg) of nicotine that enhanced cocaine in the CPP test or locomotor sensitization procedure. Also, acute nicotine exposure, whether in adolescence or adulthood, did not cause a significant increase in locomotor effect.

We have also shown that the enhancement of the cocaine reward in the CPP test by prior nicotine exposure could be replicated with d-amphetamine and morphine. In agreement with our result, Collins et al. (2004) showed that prior treatment with nicotine during early adolescence sensitized adult rats to amphetamine locomotor activation. Furthermore, mice pre-exposed to nicotine during adolescence demonstrated elevated rewarding and reinforcing effects of nicotine during adulthood (Adriani et al., 2003; Kota et al., 2009). Furthermore, adolescent nicotine exposure enhances sensitivity to cocaine and amphetamine during adolescent (Dao et al., 2011). We showed that the priming effects of nicotine on morphine also depend on the age of exposure to nicotine. Indeed, an increased behavioral response to morphine was observed when nicotine exposure occurred during early adolescence (PND 28–34) but not during adulthood.

Reversing the order of drug administration was ineffective, and adolescent cocaine pretreatment did not enhance nicotine-induced CPP. In fact, cocaine exposure in early adolescence blocked the development of nicotine preference in adult mice. This lack of cross-
sensitization could be attributable to the doses of nicotine and cocaine used and/or to differences in the pharmacokinetic properties of the drugs in adolescence. Alternatively, neuroadaptations implicated in behavioral sensitization may be different between cocaine and nicotine in adolescent animals. Interestingly, cocaine was previously reported to be a noncompetitive nicotinic antagonist in both *in vitro* and *in vivo* assays (Damaj et al., 1999; Francis et al., 2000). Furthermore, adolescent pretreatment with nicotine had no effect on expression of somatic withdrawal signs in morphine-dependent adult mice.

The cross-sensitization to these drugs of abuse (cocaine, amphetamine, and morphine) with different primary targets suggests that nicotine induces long-term molecular changes in brain circuitries that have been implicated in the rewarding and reinforcing effects of drugs of abuse, such as the mesocorticolimbic dopamine pathway (Kobb & LeMoal, 2001; Nestler, 2001), which is still developing during the adolescent period (Spear, 2000).

Furthermore, the protracted behavioral response, reported in chapters 2 through 4, suggests the involvement of delayed plastic molecular events underlying the enhancement of cocaine-rewarding effects. This speculation is in line with the recent study by Doura et al. (2010) showing that adolescent rats subjected to chronic nicotine exhibited age-specific persistent gene expression changes in the ventral tegmental area. Indeed, over 500 adolescent-specific genes showed no initial response to chronic nicotine at the end of the 2-week treatment period but showed significant up- or down-regulation 30 days after the cessation of the drug.

We have shown that nicotine-pretreated C57B/6J mice displayed a significantly higher level of cocaine-induced preference compared with mice pretreated with saline. Meanwhile, nicotine failed to prime the response to cocaine in DBA/2J mice. It is unlikely that these variations are attributable to the pharmacokinetic difference between the two strains because our
results showed that nicotine blood levels in C57B/6J and DBA/2J were similar after chronic exposure to the drug during adolescence. Differences in the expression and function of the various nAChRs subtypes or their post-receptor neurobiological signaling pathways between the two strains could play an important role. It has been shown that nicotine exposure in adolescence reduces the ability of cocaine challenge injection to enhance medial forebrain bundle stimulation-evoked DA release in the nucleus accumbens shell. The magnitude of this effect was significantly higher in DBA2/J than in C57B/J (Dickson et al., 2011). Interestingly, after exposure to cocaine and amphetamine, the ΔFosB level differed between the two strains (Conversi et al., 2011). Identifying the diversity in the behavioral, genetic, epigenetic, and molecular changes in C57B/6J and DBA/2J strains is an important goal that will help with the investigation of pathways underlying our behavioral data.

6.3. Pharmacological and molecular mechanisms involved in nicotine priming effect

The nicotine priming effects on cocaine-induced behaviors in mice were mediated by nAChRs because co-administration of mecamylamine, a nonselective nicotinic receptor antagonist, blocked the enhancement effect. In addition, DHβE and MLA co-administration during adolescence also blocked nicotine-induced enhancement of cocaine-mediated effects, suggesting that activation of α4β2* and α7 nAChRs is needed for enhancement to occur. The involvement of α4β2* nAChR subtypes is consistent with reports that higher levels of mRNAs for β2 nicotinic subunits and α4β2* high-affinity binding sites are found in adolescents than are found in adult rodents (Azam et al., 2007; Levine et al., 2007). Moreover, exposure to nicotine in mice and rats during adolescence induces a long-lasting increase in brain α4β2* nAChRs levels and functions upon reaching adulthood. For example, repeated administration of low
doses of nicotine (0.4 mg/kg i.p.) for 10 days in adolescent rats resulted in increases in mRNA expression of α5, α6, and β2 nAChRs subunits in the ventral midbrain of rats in adulthood (Adriana et al., 2003). These changes in nicotinic cholinergic receptor expression were only associated with adolescent treatments and did not occur in adult animals treated with nicotine for the same duration. A similar nicotine treatment in adolescent mice produced an increase in the α4β2* nAChRs functions in various brain regions as compared to adults (Kota et al., 2007). While these developmental changes in the expression of α4β2* nAChR levels and function may account for the unique response to nicotine in adolescent mice compared to adult mice, the exact composition of the α4β2* nAChR subtype involved in the nicotine priming effects on cocaine remains unclear because other subunits such as α5, α6, and β3 could be associated with the β2 subunits.

The blockade of nicotine priming effects by the co-administration of MLA suggests the involvement of α7 nAChRs subtypes. However, the effects of MLA could have been mediated by non-α7 nAChRs subtypes. Although it is not clear what concentrations would be achieved in the adolescent brain at the dose of 8 mg/kg of MLA, in vitro studies have shown that relatively high concentrations of MLA antagonize α6* nAChRs (Mogg et al., 2002), as well as α7 nAChRs. In line with this suggestion, the α6 subunit mRNA brain expression levels were reported to peak in the SN and VTA of rodents at PND 21 (Azam et al., 2007).

Correspondingly, data presented in Chapter five demonstrated a significant induction of ΔFosB in NAc as a result of repeated nicotine exposure in adolescence. The increase in ΔFosB level persisted during the adolescent period, but this enhancement pattern and magnitude were not found in mice exposed during adulthood. Also, no significant effect of pretreatment or age was found in PFC. Indeed, ΔFosB is a good candidate to explain our data and how nicotine
primes the brain to enhance cocaine responses in adult mice, given that chronic exposure to numerous drugs of abuse (such as nicotine, morphine, amphetamine, and cocaine) has been shown to increase the induction of ΔFosB in striatum (Nestler, 2008). The induction of ΔFosB persists for several weeks and has been reported to enhance rewarding responses to numerous drugs of abuse. ΔFosB is also involved in the long-term behavioral consequences associated with abused drugs. This is supported by studies performed on mice overexpressing ΔFosB in NAc showing enhanced sensitivity to both acute locomotor activity and rewarding effects of cocaine (Kelz et al., 1999), which suggests that expression of ΔFosB in the brain affects sensitivity to cocaine. In addition, ΔFosB overexpression in NAc increased both morphine condition place preference and morphine physical dependence (Zachariou et al., 2006). Therefore, the increase in the ΔFosB expression has a functional effect as well.

The induction of ΔFosB is long-lived but not permanent. It is degraded and returns to pre-drug levels after 1 to 2 months, suggesting that the ΔFosB protein itself does not maintain drug dependence but rather is expected to act at other targets, thus playing a role in nicotine and cocaine addictions. This speculation is in line with a study by Kelz et al. (1999) that indicated that the GluR2 subunit of the AMPA receptor is a target of ΔFosB. Furthermore, GluR2 expression was shown to increase in NAc following overexpression of ΔFosB. The study went on to eloquently show that rewarding effects of cocaine are enhanced as a result of overexpression of the GluR2 subunit, which gives another possible mechanism that would explain the data presented in chapters 2 through 4. The upregulation of receptor subunits such as GluR2 in the AMPA receptor help explain why adolescent nicotine exposure may have effects on other drugs of abuse well into adulthood. Another target gene of ΔFosB in NAc is dynorphin, or opioid peptide, which is thought to activate kappa-opioid receptors on VTA dopamine neurons.
and inhibit dopaminergic transmission, thereby decreasing reward (Nestler, 2008). Zachariou et al. (2006) have shown that the induction of ΔFosB represses dynorphin gene expression in NAc, which could contribute to the enhancement of reward mechanisms mediated by ΔFosB.

Increasing the acetylation of histones in the striatum, and specifically in the FosB promoter allow greater FosB gene expression. Histones are nuclear proteins that package DNA into chromatin and, increasing the acetylation of histones, thens unfold the DNA and opens up the promoter region that facilitates FosB gene expression (Nestler, 2008). For future studies, it may be useful to measure histone acetylation after nicotine exposure during adolescence and compare it to that in mice exposed during adulthood.

6.4 Conclusion

Adolescent tobacco dependence is a complex disease, one that starts out of curiosity, continues because of the reward response, and progresses to avoid the symptoms of withdrawal. The initiation of tobacco use in adolescence is affected significantly by social, environmental, biological, and genetic factors. During this unique developmental stage, nicotine has significant effects on future drug-taking behaviors. We demonstrated that early-adolescent mice exposed to nicotine increased cocaine CPP, locomotor activity, and locomotor sensitization in adulthood that were affected by dose, duration, method of administration, age of exposure, and mouse strain. These results suggest that early adolescence is a critical period for the behavioral plasticity induced by nicotine. Furthermore, the strain difference suggests the possible involvement of genetic factors in the effects of nicotine in adolescence.

Results from chapter 4 demonstrated that the enhancement of the cocaine reward is replicated with morphine and amphetamine. These drugs are known to affect levels of dopamine
in the brain, specifically in NAc. It is likely that the dopaminergic system is greatly affected by nicotine exposure during the early developmental period. Given that the dopamine system goes through major developmental changes during early adolescence, it is a likely candidate for the observed cross-sensitization. Yet the mechanisms underlying this cross-sensitization are still being elucidated, and additional studies would be useful for determining these pathways.

This increase in the cocaine CPP was correlated with increased ΔFosB levels in NAc, which were not seen in mice exposed to nicotine during adulthood. This suggests that repeated nicotine administration produces a unique molecular response—specifically, ΔFosB in the brain—so that, post–nicotine exposure, the brain responds differently to cocaine and other abused drugs. These data suggest that nicotine use during early adolescence may carry a greater risk than nicotine use during adulthood. Adolescent smokers may be particularly vulnerable to the risks of drugs of abuse. The work in this dissertation contributes to the further understanding of this unique developmental period.
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