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UNIVERSITY OF CALIFORNIA,
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Nicotine Craving and Withdrawal: Impact of Cigarette Smoke Constituents and Age

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Pharmacological Sciences

by

Daisy Dalila Reynaga

Dissertation Committee:
Professor Frances Leslie, Chair
Adjunct Professor James D. Belluzzi
Professor Marcelo A. Wood

2017

DEDICATION

To Brandon, Bella, and Bree who are my biggest drive and motivation in life, this is for you.

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Costello, M. R., Reynaga, D. D., Mojica, C. Y., Zaveri, N. T., Belluzzi, J. D., & Leslie, F. M. (2014). “Comparison of the reinforcing properties of nicotine and cigarette smoke extract in rats”. *Neuropsychopharmacology*, 39(8), 1843-1851. doi: 10.1038/npp.2014.31

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Presented Abstracts

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D. D. Reynaga, A. Rezk, C. Pon, D. Ghobrial, F. M. Leslie. The effects of chronic cigarette smoke extract exposure on somatic withdrawal [abstract]. In: Society for Neuroscience; 2015 October 16-21; Chicago, IL. Abstract nr 6188

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ABSTRACT OF THE DISSERTATION

Nicotine Craving and Withdrawal: Impact of Cigarette Smoke Constituents and Age

By

Daisy Dalila Reynaga

Doctor of Philosophy in Pharmacological Sciences

University of California, Irvine, 2017

Professor Frances M. Leslie, Chair

Smoking continues to be one of the leading causes of preventable death in the United States. Although the majority of smokers want to quit, most will not be successful at doing so. Despite extensive research and funding devoted to finding more efficacious pharmacotherapies for smoking cessation, current therapies are not very effective at keeping smokers abstinent for over a year. The goal of my dissertation was to establish a preclinical test that would have better face validity for the development of new pharmacological therapies for smoking cessation. Current preclinical tests of tobacco dependence use nicotine alone. Here I used an aqueous cigarette smoke extract (CSE), which contains nicotine and many other constituents of cigarette smoke, to examine its effects on cue- and drug-induced craving, factors known to influence relapse. The hypothesis that was tested was that CSE would induce greater craving and withdrawal than nicotine alone. Adult male rats that had self-administered CSE were found to reinstate to drug priming alone, unlike animals that had self-administered nicotine, which required the additional presentation of drug-associated cues. AT-1001, a functional $\alpha 3\beta 4$ nicotinic acetylcholine receptor (nAChR) antagonist, attenuated drug-primed reinstatement of CSE- and nicotine-seeking behavior. However, AT-1001 was less potent in blocking drug-

primed reinstatement in animals that had self-administered CSE than in those that had self-administered nicotine alone. This was the case even when nicotine alone was used to prime reinstatement in animals that had self-administered CSE, suggesting that prior CSE exposure had altered the functional role of this nAChR.

Another factor known to influence relapse is withdrawal to smoking. Teenagers are especially vulnerable to the effects of withdrawal. However, although adult rats show many of the same signs of withdrawal as humans following chronic nicotine exposure, adolescent rodents do not. In this study, adolescent and adult male rats were exposed for ten days to nicotine or CSE (1.5 mg/kg/day nicotine equivalent) by intravenous injection. Chronic CSE treatment resulted in greater spontaneous somatic and affective withdrawal symptoms in both adolescent and adult rats. Furthermore, adolescents treated chronically with CSE displayed major affective symptoms, as shown by an increase in anxiety-like behavior 30 days after drug withdrawal. Mecamylamine, a non-selective nAChR antagonist, was used to investigate if nAChRs are involved in the enhancement of withdrawal observed following chronic CSE treatment. Whereas mecamylamine precipitated greater somatic withdrawal in animals treated with CSE, it did not precipitate affective withdrawal.

Heavy smokers show an upregulation of nAChR through increased radioligand binding. Similarly, adult rodents show an upregulation of nAChR binding after chronic nicotine, adolescents do not. Since CSE exposure resulted in an increase in precipitated somatic withdrawal in adult and adolescent rats and also augmented nicotine-primed reinstatement, I hypothesized that chronic CSE exposure would result in enhanced nAChR binding in both adult and adolescent rats. Chronic CSE exposure results in enhanced $\alpha 4\beta 2$, $\alpha 3\beta 4$, and $\alpha 7$ nAChR binding regardless of age, in brain areas highly involved in negative aversive states of addiction.

Overall, this work provides evidence that the cigarette smoke constituents influence drug-primed craving, withdrawal, and changes in nAChR properties. Smoke constituents eliminated the protective effects that adolescent rodents display in preclinical tests of nicotine dependence, and enhanced both craving and withdrawal in adults. These findings suggest that use of CSE is a more valid way to study tobacco dependence than nicotine, and should be used in preclinical tests to assess tobacco cessation therapies.

Chapter 1

Introduction

I. NEGATIVE ASPECTS OF SMOKING

Tobacco is one of the leading causes of preventable death in the United States, killing more people annually than AIDS, alcohol, car accidents, illegal drugs, murders and suicides combined (CDC, 2014). Ninety-eight percent of tobacco users smoke cigarettes, indicating the addictive nature of cigarette smoke (CDC, 2014). Despite heightened awareness and extensive research on the detrimental consequences of smoking, over 23% of adults smoke even though the majority want to quit (Hughes et al., 1992). Of those who attempt to quit, 80% relapse within the first month, with only 3% remaining abstinent after six months without support (Hughes et al., 1992). Even with behavioral therapies and drug interventions to assist with smoking cessation, over 70% fail to remain abstinent for over a year (George and O'Malley, 2004). Smokers with smoking-related diseases, such as a myocardial infarction and cancer, report difficulty quitting despite the severe health consequences (Walker et al., 2006). These facts illustrate the strong addictive potential of cigarette smoking as well as its chronic negative impacts on public health.

Tobacco addiction is a chronic relapsing disorder characterized by a compulsive and persistent desire to smoke despite negative consequences and a desire to quit (Bauzo and Bruijnzeel, 2012; Koob and Volkow, 2010; Lynch and Sofuoglu, 2010). In humans, the major factors for relapse are craving and the negative emotional states of withdrawal (Doherty et al., 1995; Koob and Volkow, 2010; Swan et al., 1996).

Physical dependence to tobacco plays an important role in compulsive drug use (DuPont, 2010). Abrupt cessation of tobacco use in dependent individuals results in withdrawal (Fiore and Jaén, 2008). Withdrawal is a group of affective (emotional) and somatic (physical) symptoms that reflect the imbalance in brain neurochemistry created by the absence of drug (De Biasi and Dani, 2011; Paolini and De Biasi, 2011). In humans, withdrawal from tobacco use leads to somatic symptoms, such as bradycardia, insomnia, gastrointestinal discomfort, increased appetite, and weight gain (Hughes et al., 1991). Tobacco withdrawal also induces negative affective symptoms, such as irritability, depressed mood, restlessness, anxiety, increased stress, problems getting along with friends and family, difficulty concentrating, and craving for tobacco (Hughes, 1986; Hughes et al., 1991; Hughes and R., 2007). These effects emerge as quickly as 20 minutes to two hours after smoking cessation (CDC, 2004). Nicotine is completely eliminated from the body about three days after cessation, and the symptoms of withdrawal peak during this time (Hughes et al., 1991; Hughes and R., 2007). Withdrawal from smoking usually dissipates by two to four weeks of cessation, but it can take months to fully recover from the mood disturbances caused by quitting tobacco use (Gilbert et al., 1995; Hughes et al., 1991; Hughes and R., 2007). These mood disturbances are comparable in intensity to those seen in psychiatric outpatients (Hughes, 2006). The intensity of withdrawal symptoms is correlated with the likelihood of relapse (al'Absi et al., 2004; Piasecki et al., 2003a, 2003b, 2003c). Thus, an important aspect of the maintenance of smoking is prevention of withdrawal (Bruijnzeel and Gold, 2005; Koob et al., 1993).

In humans, another major factor for relapse is craving. Cigarette craving is intensified during periods of heightened stress, the presentation of drug associated cues, and smoking during a period of abstinence (Doherty et al., 1995; Nides et al., 1995). Craving induced by drug priming involves, in part, the modulation of nicotinic acetylcholine receptors (nAChRs) (Li et al., 2012a; O'Connor et al., 2010). Current cessation therapies are designed to maintain activation of these receptors to help wean off of smoking; however many are not effective past the withdrawal phase. For instance, nicotine replacement therapy (NTR) reduces withdrawal associated with cigarette abstinence, but it has not been shown to improve cessation rates after 6 months (Hughes et al., 1999; Perkins and Scott, 2008). More specific nAChR ligands, such as varenicline, that target $\alpha 4\beta 2$ and other nAChRs have been shown to be slightly more successful than NTR at a 23% success rate (Jorenby et al., 2006; Jotham W. Coe et al., 2005). This limited therapeutic efficacy may result from increased expression of $\beta 2$ - containing nAChRs in the brain, and associated craving and relapse during abstinence (Picciotto et al., 2008; Schwartz and Kellar, 1983). A better understanding of the neurobiological basis of drug-primed relapse to smoking and the differentiated involvement of nAChRs is imperative for the development of novel pharmacological targets and more efficacious smoking cessation medications.

Adolescent Smoking

The adolescent stage of development is the most critically impacted by the negative aspects of cigarette smoking. Adolescence is a period of active neuronal maturation, identified as a period of heightened vulnerability to substance abuse (Andrew

Chambers et al., 2003; Anker and Carroll, 2010). Nearly 9 out of 10 adult smokers started smoking by the age of 18, and 99% started by the age of 26 (CDC, 2014). Teen smokers show signs of dependence even before developing regular tobacco use (Breslau and Peterson, 1996; Colby et al., 2000; DiFranza et al., 2007; Zhan et al., 2012). They also tend to use more tobacco than those who begin smoking as adults, are more sensitive to withdrawal, have greater difficulty quitting, and are more likely to relapse (Kandel et al., 1992; Lai et al., 2000; Rojas et al., 1998). Hence, to fully understand smoking addiction, it is imperative to study smoking during adolescence.

Data from preclinical models of nicotine withdrawal in adolescent rats do not parallel the increased sensitivity to withdrawal and relapse found in clinical studies (O'Dell et al., 2004; V. Prokhorov, Karen Suchanek Hudmon et al., 2001). In fact, adolescent rats show fewer somatic and affective signs of withdrawal from nicotine (Laura E O'Dell et al., 2006; O'Dell et al., 2007) are less resistant to nicotine extinction (Shram et al., 2008), and are just as likely to reinstate after a nicotine-primed injection as adult rats (Shram et al., 2008). This large discrepancy between clinical and preclinical data may be due to the fact that only nicotine has been used to model tobacco dependence in these preclinical tests.

II. IS NICOTINE ALONE THE SAME DRUG AS CIGARETTE SMOKING?

Clinical Studies

Nicotine is the primary psychoactive compound in cigarette smoke and is believed to be the main component responsible for tobacco dependence (Harvey et al., 2004; Porchet et al., 1988). This is why nAChRs have been examined as the primary

mechanism underlying tobacco dependence. However, a growing clinical literature implicates the other components of tobacco smoke as additional contributors to addiction (U.S. Surgeon General, 2010). For instance, relapse rates in smokers who quit using NRT are similar to rates in those who quit without it, since it does not reduce all withdrawal symptoms (Fiore and Jaén, 2008). In contrast, de-nicotinized cigarettes do significantly reduce withdrawal and relapse (Rose et al., 2000). In a clinical study where smokers rated the subjective effects of de-nicotinized cigarettes, and intravenous nicotine or saline infusions, smokers reported that de-nicotinized cigarettes reduced their cravings and that smoking these cigarettes was significantly more satisfying and rewarding than the no smoking conditions (Rose et al., 2000). Intravenous infusions of nicotine, at doses equivalent to those obtained from cigarette smoke, were reported to reduce cravings but added feelings of dizziness and lightheadedness, with no significant satisfaction or reward (Rose et al., 2000). Clinical studies do not distinguish whether the rewarding effects of de-nicotinized cigarettes are due to a pharmacological effect or an effect of drug associated cues. Numerous studies have suggested that the cues associated with smoking are critical to reducing craving and producing satisfaction (Rose et al., 1985). However, although they include drug-paired cues, e-cigarettes have not been shown to be more effective for smoking cessation in adult smokers than nicotine replacement therapy (Bullen et al., 2013). Use of preclinical animal models is a useful approach for further evaluating these issues.

Preclinical Studies

Animal behavioral studies have been most frequently used to model the ability of different drugs to elicit addiction-related behaviors. Preclinical studies model different phases of the addiction process, such as drug initiation or acquisition, withdrawal, and chronic relapse (Lynch and Sofuoglu, 2010). As discussed below, the following studies indicate differences between nicotine alone and cigarette smoke constituent(s) in different phases of addiction:

Reward/acquisition

Self-administration Paradigm

Early demonstrations that drugs could serve as reinforcers to maintain operant behavior in laboratory animals have led to the development of self-administration as a model of human drug abuse. Self-administration is a preclinical method used to model human drug consumption and to test the reinforcing effects of drugs of abuse (Ator and Griffiths, 2003; Katz and Higgins, 2003; Shaham et al., 2003). This experimental paradigm uses operant conditioning to train an animal to self-administer a drug, and requires the animal to either press a lever or sniff a nose poke hole in order to deliver the drug intravenously via a subcutaneously implanted catheter. Reinforced lever presses or nose pokes provide a computer-controlled drug infusion if a response is made, whereas non-reinforced lever presses and nose pokes do not. Cues, either auditory or visual, are also presented at the time of drug delivery as a predictor of the rewarding effects of the drug. The frequency of reinforced responses that an animal makes correlates with the reinforcing properties of the drug.

Nicotine

While nicotine is self-administered by animals, it does not accurately model the gripping addiction faced by smokers. Very specific parameters are required for successful self-administration of nicotine alone. Standard self-administration protocols use 30 $\mu\text{g}/\text{kg}/\text{inj}$ of nicotine (free base), which is roughly equivalent to two cigarettes over a period of about 5 seconds; thus, repeated i.v. dosing at 30 or 60 $\mu\text{g}/\text{kg}/\text{inj}$, as is done in most self-administration studies (Bespalov et al., 2005; Kenny and Markou, 2001), will produce plasma concentrations much higher than in human smokers. In addition, nicotine is only weakly reinforcing in animal models compared to other drugs of abuse. When given the choice between nicotine and cocaine, rats will always choose cocaine (Manzardo et al., 2002). Furthermore, nicotine will not substitute for cocaine in self-administration tests (Mello and Newman, 2011). Therefore, these findings do not replicate the powerfully addictive nature of tobacco dependence.

Non-nicotine Constituents

Since nicotine alone does not accurately represent the complex pharmacology of tobacco smoke, researchers have recently made efforts to study smoke constituents, either in combination with nicotine or alone, to determine the potential role in behavioral reinforcement. The tobacco alkaloids are a group of compounds in tobacco smoke that are structurally related to nicotine and have direct action on nAChRs. Acute administration of nicotine combined with alkaloids found in tobacco smoke (anabasine, nornicotine, anatabine, cotinine, and myosmine) increases locomotor activity and behavioral sensitization, whereas pretreatment with tobacco alkaloids can either increase or decrease nicotine self-administration (Caine et al., 2014; Clemens et al., 2009). In a

different class of compounds, acetaldehyde, one of the most abundant constituents in cigarette smoke, will also enhance the acquisition of nicotine self-administration in adolescent rats (Belluzzi et al., 2005). In fact, rats will self-administer acetaldehyde alone when it is presented at higher doses than are found in tobacco smoke (Amit and Smith, 1985).

Cerebral and peripheral monoamine oxidase (MAO) inhibition by MAO inhibitors (MAOIs) found in cigarette smoke may inhibit the metabolism of monoamines released by nicotine such as dopamine (DA), norepinephrine (NE), and serotonin (5-HT), and add to the reinforcing effects of tobacco (Fowler et al., 1996; Lewis et al., 2007). My lab has modeled the effects of MAO inhibition on nicotine self-administration by using an irreversible and non-selective MAOI, tranlycypromine (TCP), which is not present in tobacco smoke. Rats pretreated with TCP (3 mg/kg) reliably self-administer a low dose of nicotine (10 µg/kg/infusion) that is not self-administered in controls (Villégier et al., 2007). Mechanistic studies have shown that acute treatment with TCP results in a direct increase of DA, NA, and 5-HT transmission, which also serves a critical role in the increase of nicotine reinforcement (Lotfipour et al., 2011; Villégier et al., 2007). Clorgyline, an irreversible selective inhibitor of MAO-A, has also been shown to enhance nicotine self-administration (Guillem, 2005; Guillem et al., 2006). Furthermore, pretreatment with norharmane, a beta-carboline MAOI that is naturally found in tobacco leaf and smoke, also augments nicotine self-administration (Guillem, 2005; Poindexter and Carpenter, 1962). We have recently shown that rats will reliably self-administer norharmane alone, with reinforcing effects that are additive to those of nicotine (Arnold et al., 2014). Such studies show that the non-nicotine constituents in cigarette smoke

contribute to the reinforcement properties of tobacco smoke. However, analysis of the combined action of all of the components in cigarette smoke may provide a more valid model of tobacco dependence.

Tobacco Extracts

To study the collective effects of the thousands of constituents in cigarette smoke, researchers have used extracts from tobacco leaves, whole tobacco smoke extracts, or smoke exposure in specialized smoking chambers (Harris et al., 2010; Small et al., 2010). Extracts from tobacco leaves are not a model of cigarette smoking, since they do not model combustion products, and passive exposure to cigarette smoke is unsuitable for use in self-administration test models (Dworkin & Dworkin, 1995). Aqueous extracts from whole tobacco smoke form a solution that animals can actively self-administer, and is the approach used in the current proposal.

Tobacco particulate matter (TPM) is an extract produced from the particulate phase (or “tar” phase) of tobacco smoke that it is evaluated for its matching dose of nicotine and non-nicotine constituents harmaline and norharmaline (MAOIs) (Brennan et al., 2015; Danielson et al., 2014; Lewis et al., 2007). Responding in self-administration tests of TPM made from cigarettes follows a flat dose response curve much like nicotine. However, self-administration of TPM made from roll your own tobacco (RYO) was higher at 15 and 30 $\mu\text{g}/\text{kg}/\text{infusion}$ (Brennan et al., 2015). In addition, TPM made from cigarettes did not yield higher progressive ratio responding; however (RYO) TPM (15 and 30 $\mu\text{g}/\text{kg}/\text{infusion}$) did (Brennan et al., 2015). The pharmacological mechanisms underlying TPM and nicotine self-administration were also different. Whereas the 5-HT_{2A/C} receptor antagonist ketanserin decreased responding for nicotine it did not for

cigarette TPM. In addition, mecamylamine, a nonselective nAChR antagonist, did not inhibit TPM self-administration to the same extent as that of nicotine. Together these studies show that including the non-nicotine constituents from tobacco smoke may increase the reinforcing properties of nicotine; however this effect is highly dependent on the origin of the non-nicotine constituents being studied. TPM is produced from the particulate phase of tobacco smoke that consists of mainly tar, nicotine, and water. However many constituents exist in dynamic equilibrium between the particulate and gas/vapor phase. Ninety-five percent of the mass of mainstream smoke exists in the gas phase (Baker, 2006; Borgerding and Klus, 2005). Therefore, studying the effects of non-nicotine constituents in whole mainstream cigarette smoke would serve as a more relevant model of smoking.

Our lab seeks to do this with cigarette smoke extract (CSE), which is made by bubbling whole mainstream cigarette smoke through a saline solution (Costello et al., 2014; Gellner et al., 2016). This makes an aqueous solution that both adult and adolescent rats readily self-administer intravenously and hence can be used in behavioral test of drug addiction (Costello et al., 2014; Gellner et al., 2016). Rats self-administered CSE at low doses of nicotine that yield blood levels close to that of human smokers (Costello et al., 2014; Gellner et al., 2016). The minimum reinforcing CSE doses were much lower than the standard 30 µg/kg/infusion nicotine dose used in most rat self-administration studies (Corrigall and Coen, 1989; Donny et al., 1995) and are near the amount of nicotine a smoker receives in a single puff of a cigarette (Miller et al., 1977; Rose and Corrigall, 1997). However, CSE did not exhibit higher progressive ratio responding in adult rats, showing that CSE is more potent but not more reinforcing than

nicotine alone (Costello et al., 2014). Analysis of nicotine content in plasma and brain tissue revealed no differences between adult animals that self-administered CSE or nicotine, suggesting that differing pharmacokinetic profiles do not account for this difference in potency (Costello et al., 2014). Antagonist studies reveal that nAChR activation is required for the reinforcing properties of both drugs. Mecamylamine and varenicline reduced responding for both CSE and nicotine to the same degree (Costello et al., 2014). However, AT-1001, an $\alpha 3\beta 4$ nAChR partial agonist (Toll et al., 2012), was less effective at reducing responding for CSE than nicotine (Costello et al., 2014). Collectively these findings from the acquisition, maintenance, and progressive ratio experiments show that smoke extracts are more potent than nicotine alone. The antagonist studies suggest that the differences in reinforcement might be due to activation of differing neuronal mechanisms.

Craving/relapse

Extinction-reinstatement Paradigm

The self-administration extinction-reinstatement paradigm is used to model human relapse in laboratory animals. Reinstatement has face validity as a model of human addiction since the triggers that cause relapse and craving in humans can reliably reinstate drug-seeking behavior in laboratory animals (Shaham et al., 2003). Just like in humans, in animal models, the two most effective events for reinstating drug-seeking behavior after both short-term and long-term drug free periods are re-exposure to the drug itself or exposure to a brief period of stress (Stewart et al., 1984). During extinction, the drug and any drug-associated cues are removed. After a pre-determined extinction

criterion is reached, drug-seeking behavior is reinstated by the presentation of drug-associated cues, a priming injection of drug, or a stressor (usually achieved with the use of the chemical stressor, yohimbine) (Costello et al., 2014). Reinstatement is then evaluated by measuring responding at the previously reinforced lever or nose-poke, even when drug is not delivered.

Nicotine

Although the rate of relapse and the abuse liability of tobacco are comparable to or greater than other drugs of abuse, such as stimulants and opiates (Caggiula et al., 2001), animal models of nicotine reinstatement do not readily predict the difficulty smokers experience in maintaining abstinence. The need for presentation of nicotine-associated environmental cues in animal models of self-administration and reinstatement is greater than for other abused drugs (Chaudhri et al., 2007, 2006; Sorge et al., 2009). Rats that are trained to lever press for nicotine will dramatically reduce their responding if the drug-associated cues are removed (Caggiula et al., 2001). After nicotine abstinence, both drug- and stress-induced reinstatement of nicotine seeking is enhanced by presentation of drug-associated cues (Feltenstein et al., 2012; Schenk et al., 2008). In fact, a priming dose of nicotine will not reinstate nicotine-seeking behavior unless it is paired with drug-associated cues (Schenk et al., 2008). This lack of representation of the gripping addictive nature of smoking may be due to the absence of the non-nicotine smoke constituents in the experimental model.

Cigarette Smoke Extract

Our evaluation of extinction-reinstatement of CSE-seeking behavior is the first and only model to represent smoking in paradigms of relapse. Responding for CSE was

more persistent during the first day of extinction than for an equivalent dose of nicotine (Costello et al., 2014), indicating that constituents in CSE are adding to the reinforcing value of nicotine. Furthermore, unlike nicotine alone, stress-induced reinstatement of CSE seeking was robust without the presence of cues (Costello et al., 2014). In addition, animals that self-administered CSE were more sensitive to stress-induced reinstatement than animals that self-administered nicotine alone (Costello et al., 2014), making it a more fitting model for relapse of smoking, as stress is a significant trigger of relapse in humans.

Dependence/withdrawal

Nicotine

Rodent models of chronic nicotine exposure show many of the same symptoms of withdrawal as humans. In rodents, discontinuation of chronic nicotine administration, either spontaneously or precipitated by the administration of nAChR antagonists, such as mecamylamine, results in development of characteristic somatic and affective withdrawal symptoms (Malin et al., 1994, 1992). The distinction between somatic and affective symptoms originated from the notion that the somatic signs reflect mainly peripheral mechanisms in contrast with the affective symptoms, which are produced by central mechanisms (Markou et al., 1998; Watkins et al., 2000). However, there is increasing evidence that somatic signs may have a central component that reflects a dysphoric state of heightened irritability (Malin and Goyarzu, 2009; Salas et al., 2009a).

Somatic signs of nicotine withdrawal include abdominal constrictions, facial fasciculation, increased eye blinks, and ptosis (Malin et al., 1994, 1992). Affective signs

of withdrawal include anxiety, anhedonia, conditioned fear, conditioned place aversion (CPA), hyperalgesia, and depression (Damaj, 2003; De Biasi and Salas, 2008; Winter et al., 2011). Affective signs in rodents can be measured by intracranial self-stimulation (ICSS; as a measure of anhedonia), CPA, elevated plus maze (EPM), light-dark box activity, and open field locomotion, where the latter three serve as measures of anxiety (Costall et al., 1989; Pellow et al., 1985; Treit and Fundytus, 1988). Nicotine withdrawal, when precipitated in rodents by the administration of a nAChR antagonist, produces a weak CPA (Suzuki et al., 1996; Watkins et al., 2000). Furthermore, upon withdrawal from relatively high doses of nicotine, the animals will spend less time in the open arm of the EPM than saline treated rats (Damaj, 2003; Treit and Fundytus, 1988), and will exhibit an increase in thigmotaxis, or time spent in the periphery of the open field (Treit and Fundytus, 1988; Tzavara et al., 2002). Collectively, these studies show that nicotine withdrawal induces somatic and negative affective states, although these states are highly dependent on the dose of nicotine previously administered.

Non-nicotine Constituents

Because withdrawal reflects the imbalance neurocircuitry that results in the absence of a drug in a dependent individual. to study withdrawal to smoking, preclinical models should include the contents of whole cigarettes smoke. The only method to date to study withdrawal from whole tobacco smoke in animal models is via passive inhalation. Chronic exposure to tobacco smoke has been shown to induce both physical and psychic dependence, as compared to nicotine vapor or non-smoke exposed controls, but only at extensive schedules of exposure (Ponzoni et al., 2015; Small et al., 2010). MAOIs in cigarette smoke may mediate the manifestation of withdrawal, phenelzine, a

non-selective MAOI, prolongs the duration of nicotine withdrawal, as measured by conditioned place aversion (Guillem et al., 2008; Malin et al., 2013).

Age Effects in Withdrawal: Nicotine vs Cigarette Smoke

There are major discrepancies between the findings of human and animal adolescent withdrawal studies. Whereas human adolescents have an increased sensitivity to the effects of smoking cessation (O'Dell et al., 2004; V. Prokhorov, Karen Suchanek Hudmon et al., 2001; Zhan et al., 2012), rodent adolescents are less sensitive than adults to nicotine withdrawal effects both somatic and affective (Laura E O'Dell et al., 2006; O'Dell et al., 2004; Smith et al., 2006; Hugo A Tejada et al., 2012). One possible reason for this discrepancy may be that the animals are exposed to nicotine alone and not to other non-nicotine constituents also found in cigarette smoke. Recently, a group found that adolescent rats exposed to cigarette smoke via passive inhalation exhibited increased anxiety-like behavior and increases in locomotor activity during withdrawal (De la Peña et al., 2016). This demonstrates how inclusion of cigarette smoke constituents can influence the withdrawal syndrome.

III. ROLE OF NACHRS IN WITHDRAWAL, CRAVING, AND RELAPSE

Nicotine exerts its actions through the activation of nAChRs that also respond to the endogenous neurotransmitter acetylcholine (ACh) (Picciotto et al., 2012). AChRs are ligand gated cation (K^+ , Na^+ , Ca^{2+}) channels, found centrally and peripherally, composed of hetero-pentameric combinations of $\alpha 2-6$ with $\beta 2-4$ subunits, or homo-pentameric assemblies of $\alpha 7-10$ subunits (Dani and Bertrand, 2007). In the brain, nAChRs are

preferentially located at pre-terminal and pre-synaptic sites where, upon activation, ion influx cause local depolarization or activation of Ca^{2+} dependent mechanism, regulating the release of both excitatory and inhibitory neurotransmitters (Albuquerque et al., 2009; Jensen et al., 2005). Although nicotine binds only to the α subunit, all subunits contribute to the receptor's unique signaling, affinity, efficacy, desensitization, and channel permeability. The unique pharmacology as well as neuroanatomical location of nAChRs contribute to each subtypes' unique roles in nicotine dependence (Cippitelli et al., 2015b; De Biasi and Salas, 2008; Stoker and Markou, 2015, 2013; Zaveri et al., 2015). The $\alpha 4\beta 2$ nAChRs are the most ubiquitously expressed central nAChR subtype; they possess high-affinity to nicotine, and rapid desensitization upon activation (Gotti et al., 2009; Millar and Gotti, 2009). Homomeric $\alpha 7$ nAChRs are the next most abundant and have relatively lower nicotine affinity, higher Ca^{2+} permeability, and slower desensitization kinetics than $\alpha 4\beta 2$ -containing nAChRs (Clarke et al., 1985; Dajas-Bailador and Wonnacott, 2004). Overall all, $\alpha 4\beta 2$ and $\alpha 7$ nAChRs have been the most implicated in nicotine addiction; however, recent studies have found important contributions of other subtypes such as $\alpha 3\beta 4$ nAChRs (for review see Leslie, Mojica, & Reynaga, 2013). $\alpha 3\beta 4$ nAChRs have lower affinity to nicotine than $\alpha 4\beta 2$ and $\alpha 7$ nAChRs, do not readily desensitize after activation (Ciuraszkiewicz et al., 2013; Nelson and Lindstrom, 1999). In the brain the receptor is highly expressed in the medial habenula (MHb), interpeduncular nucleus (IPN) and the pineal gland, areas that play a crucial role in self-administration behavior and modulate negative reward (Fowler et al., 2011; Perry et al., 2002; Salas et al., 2010). The unique characteristics of the $\alpha 3\beta 4$ nAChR make it a novel target with great therapeutic potential to treat tobacco dependence.

$\alpha 3\beta 4$ as a New Target to Attenuate Relapse

Tobacco dependence is extremely difficult to treat. For instance, varenicline, the most effective drug on the market only increases the odds of quitting by 30% (Alpert et al., 2013). The low rates of quitting success for these therapies, coupled with the significant toxic effects of smoking, has prompted continued efforts to discover new targets for smoking cessation.

The $\alpha 3\beta 4$ nAChR is showing promise as a new target for tobacco dependence treatment. Gene wide association studies have shown polymorphisms in the gene cluster encoding for the $\alpha 3$ - $\alpha 5$ - $\beta 4$ nAChR subunits are associated with an increased risk for tobacco dependence (Berrettini et al., 2008). The $\alpha 3\beta 4$ nAChR controls acetylcholine release in the fasciculus retroflexus, the pathway connecting the MHb and IPN (Grady et al., 2009). Blocking cholinergic transmission in either the MHb or IPN is sufficient to precipitate somatic signs of nicotine withdrawal (Salas et al., 2009b).

Compounds that interact with the $\alpha 3\beta 4$ nAChR have an effect on nicotine reward and reinforcement in animals. For instance, 18- Methoxycoronaridine (18-MC), a $\alpha 3\beta 4$ nAChR antagonist, reduces nicotine self-administration in rats when administered systemically and directly into the MHb (Glick et al., 2006). Another $\alpha 3\beta 4$ nAChR antagonist, AuIB, blocks nicotine CPP (Jackson et al., 2013). AT-1001, a highly selective partial agonist of $\alpha 3\beta 4$ nAChR potently blocked self-administration for nicotine (Toll et al., 2012). In a dose response experiment, AT-1001 also reduced responding for CSE, however it did not do so to the same degree as with nicotine (Costello et al., 2014). Although much work has gone into investigating the role of $\alpha 4\beta 2$ and $\alpha 7$ nAChRs in drug-primed relapse, investigation of $\alpha 3\beta 4$ involvement has been more limited. A current

study however showed that AT-1001 drug-primed reinstatement of nicotine- seeking in rats (Cippitelli et al., 2015a, 2015b; Toll et al., 2012). Together, targeting of $\alpha 3\beta 4$ nAChRs shows promising as a smoking cessation therapy.

Upregulation of nAChRs in Tobacco Dependence

Heavy smokers show increased radioligand binding to nAChRs (Brody et al., 2003; Marks et al., 1983; Schwartz and Kellar, 1983). This “upregulation” of receptor binding has been implicated as a possible mechanism underlying the addictive potential of nicotine. Increases in radioligand binding are also observed in preclinical models of in-vitro and in-vivo chronic nicotine exposure. These studies reveal that the patterns of upregulation are subtype, brain area, and nicotine dose and treatment paradigm dependent (for review see Henderson & Lester, 2015). Upregulation of nAChR binding has not been thoroughly studied in preclinical models of chronic cigarette smoke exposure. Recently, a group showed similar levels of receptor binding to $\alpha 4\beta 2$ nAChRs after chronic second-hand cigarette smoke exposure and e-cigarette vapor in adult mice (Ponzoni et al., 2015). These findings may be unique to the method of exposure as an extensive 7-week treatment was required to see an effect while $\alpha 7$ nAChRs did not upregulate as expected (Ponzoni et al., 2015).

Age Differences in nAChR Upregulation

The role of nAChRs in mediating age differences in nicotine dependence and withdrawal is not well understood. Studies have compared changes in nAChR expression following nicotine exposure in adolescent and adult rats. This work has revealed that the changes in nAChRs are age-, receptor subtype-, and region-dependent (Counotte et al.,

2012; Doura et al., 2008; Trauth et al., 1999). In nicotine-naive rats, receptor binding is higher in adolescents than adults (Doura et al., 2008). Furthermore, ligand binding to $\alpha 7$ and $\alpha 4\beta 2$ nAChRs is increased after chronic nicotine exposure to a greater extent in adults than in adolescents (Doura et al., 2008; Slotkin et al., 2004; Trauth et al., 1999). Thus, enhanced nAChR function and resulting modulation of activity in the VTA, coupled with a resistance to receptor up-regulation, may explain why adolescents display resistance to nicotine withdrawal (Badanich & Kirstein, 2004). Unique age effects in upregulation after chronic cigarette smoke exposure has been underexplored.

IV. CONCLUSION

I believe that in order to obtain more effective smoking cessation therapies, current preclinical research methods must be improved. My lab's efforts to improve preclinical models has prompted us to investigate the addiction potential of CSE on preclinical tests of self-administration and stress-induced reinstatement. I have now compared the effects of drug-primed reinstatement on nicotine- and CSE-seeking behavior. Using this model, I have also tested the effect of AT-1001, a novel cigarette smoke cessation therapy that targets $\alpha 3\beta 4$ nAChRs, to attenuate drug-primed reinstatement. Additionally, I have investigated the effects of passive intravenous administration of CSE on physical and affective aspects of withdrawal. To date, CSE has not been used to model withdrawal in adolescence, a gap that I have addressed in my studies. I specifically tested the hypothesis that the non-nicotine constituents of CSE will induce greater dependence than nicotine alone in adult and adolescents rats, making it a more valid tool to study tobacco dependence than nicotine alone. Furthermore, I explored

differences in nAChR pharmacology as a way to analyze the possible mechanism of CSE's impacts on addiction. I believe that my findings will lead to the development of improved treatments for smoking cessation and help better preserve the health of our youth and the general public.

Chapter 2

Role of $\alpha 3\beta 4$ Nicotinic Acetylcholine Receptors in Cue- + CSE- and Nicotine-primed Reinstatement of Drug-seeking Behavior

INTRODUCTION

Tobacco addiction is a chronic relapsing disorder characterized by a compulsive and persistent desire to smoke, despite negative consequences and a desire to quit (Bauzo and Bruijnzeel, 2012; Koob and Volkow, 2010; Lynch and Sofuoglu, 2010). Of those who attempt to quit without support, 80% relapse within the first month, with only 3% remaining abstinent after six months (Hughes et al. 1992). Even with behavioral therapies and drug interventions to assist with smoking cessation, over 70% fail to remain abstinent for over a year (George and O'Malley, 2004). Thus, it is clear that cigarette smoking is highly addictive and current cessation therapies are largely ineffective.

In humans, a major factor for relapse is craving. Cigarette craving is intensified during periods of heightened stress, the presentation of drug associated cues, and smoking during a period of abstinence, also known as drug-priming (Doherty et al., 1995; Nides et al., 1995). Relapse induced by cues or drug- priming involves, in part, the modulation of nAChRs (Le Foll et al., 2012.; Li et al., 2012; O'Connor et al., 2010). Current cessation therapies are designed to maintain activation of these receptors to help wean smokers from smoking; however, many are not effective past the withdrawal phase. It has been proposed that the limited therapeutic efficacy of current cessation drugs that target $\alpha 4\beta 2$ nAChRs, such varenicline, may be due to increased expression of $\beta 2$ -containing nAChRs in the brain resulting in craving and relapse during abstinence

(Picciotto et al., 2008; Schwartz and Kellar, 1983). A better understanding of the neurobiological basis of - and drug-induced relapse to smoking and the differentiated involvement of nAChRs is imperative for the development of novel pharmacological targets and more efficacious smoking cessation medications.

Recent studies have demonstrated the importance of $\alpha 3\beta 4$ nAChRs in tobacco addiction and as a potential new target for smoking cessation. Gene-wide association studies reveal that polymorphisms in the gene cluster encoding $\alpha 3\text{-}\alpha 5\text{-}\beta 4$ nAChR subunits are associated with an increased risk for tobacco dependence (Schlaepfer et al., 2008). Although much work has gone into investigating the role of $\alpha 4\beta 2$ and $\alpha 7$ nAChRs in cue- and drug-induced relapse, investigation of $\alpha 3\beta 4$ involvement has been more limited. Recent studies, however, have shown that AT-1001, a selective partial agonist of $\alpha 3\beta 4$ nAChRs, blocks self-administration and cue- + nicotine-primed reinstatement of nicotine seeking in rats (Cippitelli et al., 2015a, 2015b; Costello et al., 2014; Toll et al., 2012). Thus, $\alpha 3\beta 4$ nAChRs may play an important role in nicotine craving, and targeting of these nAChRs may show promise for smoking cessation therapy.

Use of nicotine alone in preclinical tests of drug-primed reinstatement does not readily predict the difficulty smokers experience in maintaining abstinence (Chaudhri et al., 2007, 2006; Feltenstein et al., 2012; Schenk et al., 2008; Sorge et al., 2009). A growing body of preclinical literature has shown that non-nicotine constituents in tobacco smoke also contribute to nicotine addiction (United States Department of Health and Human Services, 2014), and therefore should be included in screening of potential pharmacological cessation therapies. To this end, we have created an aqueous extract of cigarette smoke extract, CSE (Costello et al., 2014; Gellner et al., 2016). When compared

to an equivalent dose of nicotine alone, animals that self-administered CSE were more sensitive to stress-induced reinstatement, and showed robust reinstatement without the presence of drug associated cues, suggesting that it may represent an improved model to assess relapse to smoking (Costello et al., 2014). Further, AT-1001 was less effective at reducing self-administration of CSE than of nicotine (Costello et al., 2014), providing further evidence for the need of cigarette smoke constituents to be included in preclinical models of smoking.

The purpose of the present study was to assess the effects of cigarette smoke constituents on nicotine craving and relapse vulnerability and to investigate the role of $\alpha 3\beta 4$ nAChRs in this behavior. Here I compare cue- and drug-induced reinstatement in animals that have previously self-administered CSE or nicotine, and the antagonistic effects of AT-1001 on this behavior. The main hypothesis guiding these experiments is that animals that self-administered CSE will show enhanced reinstatement as compared to animals that self-administered nicotine alone, and that this will result in a reduced potency of AT-1001 at attenuating cue- + drug- induced reinstatement.

MATERIALS AND METHODS

Drugs

Nicotine hydrogen tartrate (Sigma, St. Louis, MO) was dissolved in sterile saline and adjusted to pH 7.2-7.4. All nicotine doses were calculated as free base. CSE was created daily by bubbling the smoke from commercial cigarettes (Camel unfiltered, R.J. Reynolds Co.) through sterile saline, based on our previous methods (Costello, et al., 2014). Eight cigarettes were smoked through 35 ml of saline solution (35 ml puffs over

2s, repeated every 30s) and the final solution was adjusted to pH 7.2-7.4. All CSE doses were defined by the nicotine content in the solution determined by GC-MS after a nicotine extraction, based on Jaycob et al., 1981. Mecamylamine HCl (Tocris Bioscience, Bristol, UK) was dissolved in sterile saline. AT-1001 (kindly provided by Dr. Nurulain Zaveri, Astrea Therapeutics, Mountain View, CA) was dissolved in 97% (0.5% concentration in water) hydroxypropylcellulose, 2% DMSO and 1% 0.1 M HCl.

Animals

Adult male Sprague-Dawley rats (300-325g; Charles River Labs in Hollister, CA) arrived at postnatal day (P)81 and were housed in an AALAC-accredited vivarium on a 12-h light/dark cycle (1900 to 0700 h). All procedures were in compliance with NIH guidelines and were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine. After two days acclimation to the vivarium, animals were handled for two minutes daily before testing began. Behavioral testing was conducted 7 days per week. Animals had dietary restriction to maintain 85% of their free-feeding body weight during food training and 95% during the remainder of the study.

Food training

Animals were trained once per day in a 30 min session to lever press for food pellets (45 mg rodent purified diet; Bio-Serv, Frenchtown, NJ) in lever pressing operant testing chambers (Med Associates, St. Albans, VT) based on Liechti et al., 2007. One wall of the chamber contained two levers, a cue light over each, and a house light. At the beginning of the session, the house light was illuminated and responses at the reinforced (R) lever resulted in reward and an illumination of the cue light over that lever.

Responses at the non-reinforced (NR) lever had no consequence, but were recorded as a measure of nonspecific activity. The animals started at an FR1TO1 (fixed-ratio 1, 1 s timeout) schedule of reinforcement, followed by FR1TO10, FR2TO20 and finally FR5TO20, progressing upon earning 50 reinforcers.

Surgery

After food training, animals were anesthetized with equithesin (0.0035ml/g body weight) and implanted with indwelling jugular vein catheters based on previously published methods (Belluzzi et al, 2005). During the 2-3 day recovery period, and for the remainder of the study, animals were flushed daily with heparinized saline solution (1ml of 1000 units/ml heparin into 30 ml bacteriostatic saline). Catheter patency was verified by infusing 0.1ml of propofol (Abbott Laboratories, Chicago, IL) for rapid anesthesia after stabilization of self-administration was achieved, before the start of the extinction phase.

Drug self-administration and extinction

As in Costello et al., 2014, animals self-administered nicotine or CSE (15 μ g/kg/infusion nicotine content) at a FR5 schedule for 1-hour session/day for a minimum of 10 days, or until they reached stable responding (reinforced responses (R) within 20% of the mean over the last 3 days; $R \geq 2 \times$ non-reinforced (NR) responses; $R \geq 6$). After reaching stable responding, extinction-reinstatement testing began. During extinction, animals were placed in the same operant testing chambers; the animals were not connected to the infusion tubing, the house light remained on, and responses on the levers

had no consequence. Extinction sessions were 1hr per day for a minimum of 5 days, or until responding was reduced to 20% of baseline.

Cue- and drug-induced reinstatement

After reaching extinction criteria, one group of CSE and nicotine animals were triggered to reinstate drug-seeking behavior using five reinstatement conditions (given in a within-subjects counter-balanced design): cues, CSE- prime alone, nicotine- prime alone, CSE- prime paired with cues, and nicotine-prime paired with cues. Presentation of cues consisted of cue light illumination, and all i.p drug prime injections contained nicotine (0.15 mg/kg) or CSE with equivalent nicotine content, given immediately before the test. Between reinstatement tests, animals were returned to extinction conditions for a minimum of two days, or until extinction criteria were met. Reinstatement is defined as a significant increase in responding from extinction.

$\alpha 3\beta 4$ nAChR blockade of drug- + cue-induced reinstatement

Following extinction, separate groups of animals that had self-administered nicotine or CSE were treated with AT-1001 (0, 0.75, 1.5, and 3 mg/kg; s.c.) before reinstatement testing (given 10 minutes before the test in a within subjects Latin-square design as in Toll et al., 2012). Drug-primed + cue reinstatement was done with a priming dose of CSE or nicotine (0.15 mg/kg) in animals that had self-administered CSE, or a priming dose of nicotine (0.15 mg/kg) in animals that had previously self-administered nicotine. Between reinstatement tests, animals were returned to extinction conditions for a minimum of two days, or until extinction criteria were met. All animals repeated a

vehicle dose of AT-1001 at the end of the study to confirm reinstatement was still taking place. Animals that did not pass a reinstatement criterion of 40% or more from the last FR5 responding at the vehicle dose were excluded from the study.

Data analysis

Self-administration and extinction responding was analyzed with a 3-way ANOVA on Drug x Day x Responding with repeated measure on Day and Responding. To normalize data, both the extinction and reinstatement data were analyzed as a percentage of baseline responding, calculated as: (Test day responding/Last day of FR5 responding) x 100. Mean responding was analyzed by a 2-way ANOVA on Drug x Condition or Drug x AT-1001 Dose, with repeated measures on Reinstatement Condition or AT-1001 Dose. Significant main effects were analyzed further with 2- or 1- way ANOVAS and bonferonni corrected paired t-test or unpaired t-tests.

RESULTS

Cue- and drug-induced reinstatement

During the initial self-administration and extinction phases, no significant drug differences were observed (Fig. 2.1 A, B). Both drug groups had significantly higher reinforced (R) responding than non-reinforced (NR) responding daily during self-administration and extinction ($p = 0.000$) (Fig. 2.1 A, B). During extinction, both drug groups had significantly lower responding on days 2-5 than the first day of extinction ($p = 0.000$) (Fig. 2.1 B).

For the reinstatement test, two-way ANOVA revealed significant main effects of Reinstatement Condition ($F_{5,125} = 10.248$; $p = 0.000$) and Drug ($F_{1,25} = 6.125$; $p = 0.02$) (Fig. 2.1). Consistent with prior literature (Feltenstein et al., 2012; Schenk et al., 2008), a priming injection of nicotine reinstated drug-seeking behavior in rats that had previously self-administered nicotine, but only if drug-associated cues were present ($p = 0.05$; Fig. 2.2). A priming injection of CSE also reinstated drug-seeking behavior in these animals when presented with cues, even though they had not self-administered CSE ($p = 0.015$) (Fig. 2.2). Animals that had self-administered CSE reinstated responding with priming injections of both CSE and nicotine, with ($p = 0.01$, $p=0.03$) and without ($p = 0.03$) the presentation of drug-associated environmental cues (Fig. 2.2). Animals that had self-administered CSE showed significantly higher responding than those that self-administered nicotine after both cue- induced and nicotine-primed reinstatement ($p = 0.027$ and 0.026 , respectively).

$\alpha 3\beta 4$ nAChR blockade of reinstatement

In order to test the hypothesis that prior self-administration of CSE altered the functional role of $\alpha 3\beta 4$ nAChRs in reinstatement, the effect of AT-1001 on drug- + cue-primed reinstatement of drug-seeking behavior was examined. Two-way ANOVA of AT-1001 inhibition of drug- + cue-primed reinstatement revealed significant main effects of Reinstatement Condition ($F_{4,128} = 53.178$; $p = 0.000$) and Drug ($F_{1,32} = 4.195$; $p = 0.024$) (Fig. 2.3). Significant AT-1001 Dose x Drug interactions were also observed ($F_{8,128} = 2.913$; $p = 0.005$). All animals significantly reinstated to drug- + cue when saline was administered instead of AT-1001 ($p = 0.000$). AT-1001 dose-dependently attenuated

reinstatement in animals that had self-administered nicotine or CSE, although higher doses of AT-1001 were needed for the latter. The 0.75 mg/kg dose of AT-1001 fully attenuated reinstatement of nicotine-seeking in animals that had self-administered nicotine alone ($p = 0.000$) but not CSE-seeking in animals that had self-administered CSE and were primed with either CSE or nicotine as they still showed significant increases in responding than extinction ($p = 0.028$, $p = 0.007$ respectively). At this AT-1001 dose, animals that had self-administered CSE and were primed with either nicotine or CSE reinstated significantly more than animals that had self-administered nicotine and were primed with nicotine ($p = 0.013$). At higher doses, AT-1001 significantly attenuated reinstatement in all groups (Fig. 2.3).

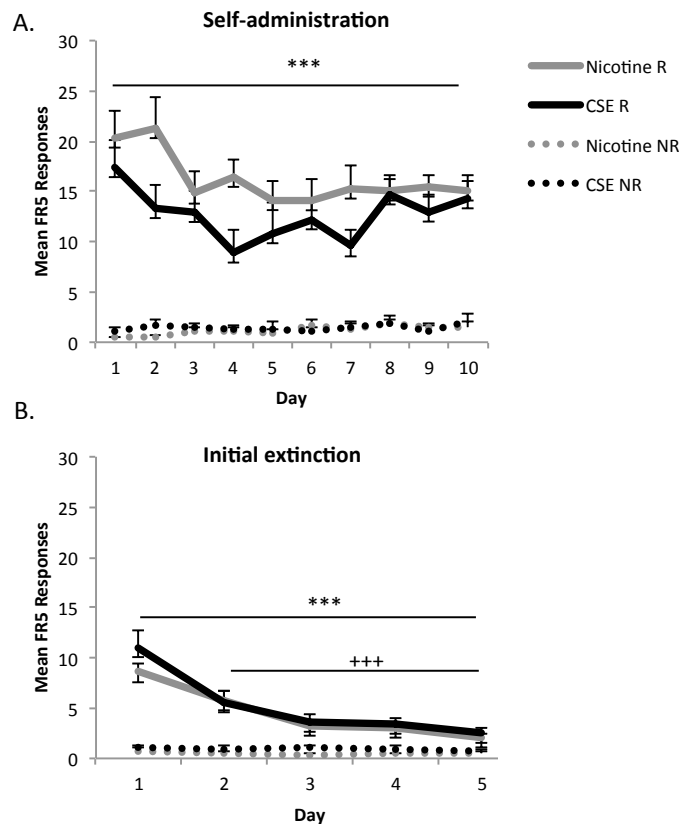


Figure 2.1. There are no drug differences in self-administration and extinction responding. *** = $p \leq 0.001$ R vs NR; +++ = $p \leq 0.001$ vs Day 1 R responding. $n = 13-14$ per group.

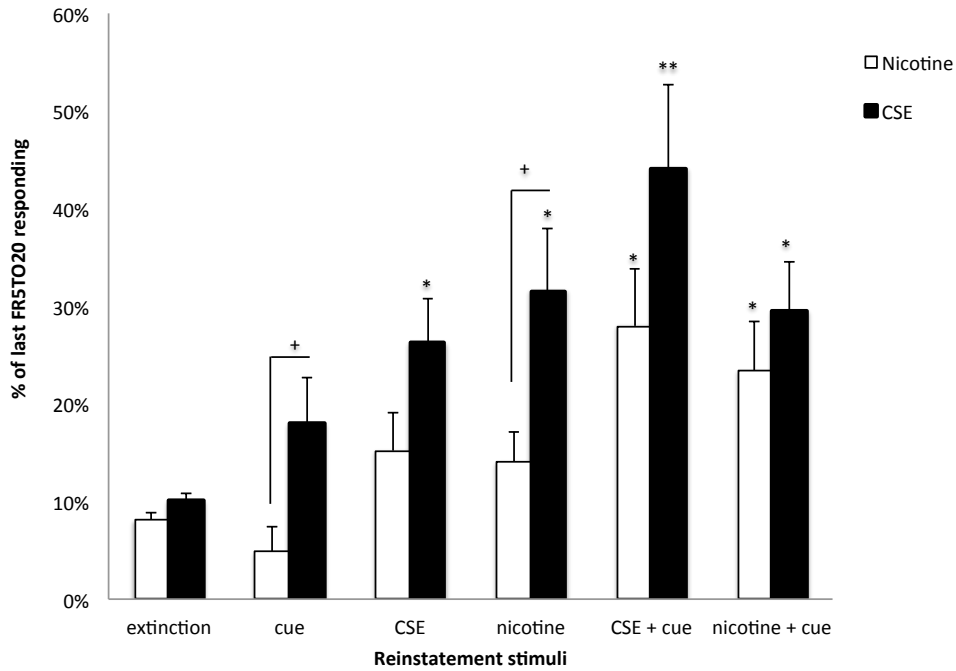


Figure 2.2. Animals that self-administered CSE are more sensitive to drug-primed reinstatement than those that self-administered nicotine alone. * = $p \leq 0.05$ vs. extinction; + = $p \leq 0.05$ vs. nicotine. n = 13-14 per group.

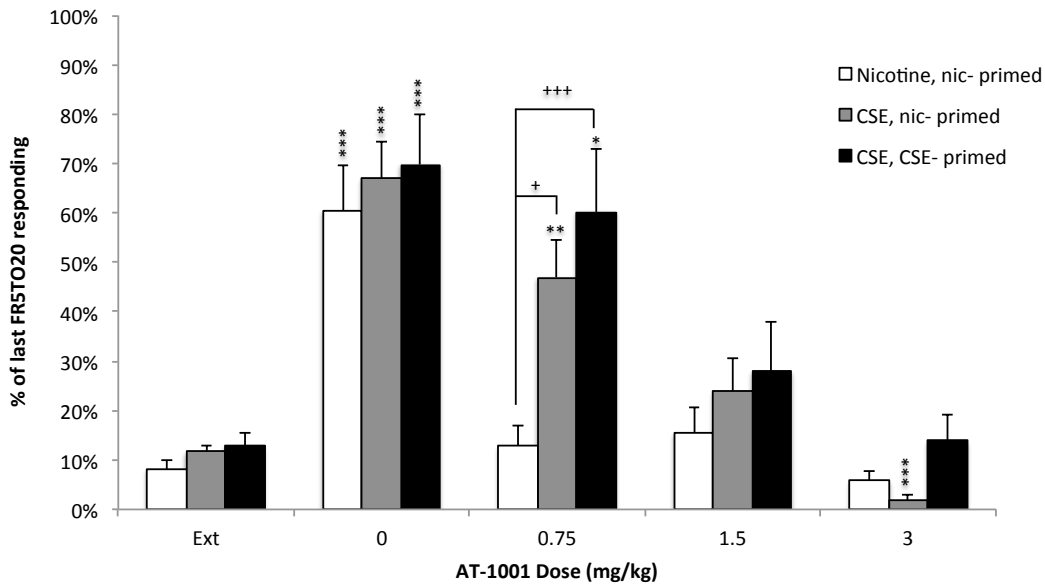


Figure 2.3. AT-1001 dose-dependently attenuates CSE- and nicotine-primed reinstatement (paired with cues) with higher potency in animals that previously self-administered nicotine at the 0.75 mg/kg dose. *** = $p \leq 0.001$; * = $p \leq 0.05$ vs. extinction; +++ = $p \leq 0.001$, + = $p \leq 0.05$ vs. nicotine. n = 10-13 per group.

DISCUSSION

This is the first set of experiments investigating the effects of CSE self-administration on drug- and cue- reinstatement. The reinstatement procedure is a widely used preclinical paradigm to study “relapse”. A similarity in factors, such as drug priming and the presentation of drug-associated cues, that induce relapse in humans and reinstatement in animals, suggest good etiological validity for the reinstatement model. However, animal models of nicotine reinstatement do not readily predict the difficulty smokers experience in maintaining abstinence. For instance, as shown here and by others, animals that had previously self-administered nicotine required the presentation of drug-associated cues to reinstate after drug-priming (Chaudhri et al., 2007; Sorge et al., 2009). However, unlike with nicotine, animals that had self-administered CSE reinstated following drug priming without drug-associated cues, although the presentation of cues alone did enhance CSE-seeking behavior. This shows that repeated CSE exposure sensitized animals to the effects of both drug-priming and drug-associated cues. This suggests that the discrepancy between the reinforcing potency of nicotine in preclinical tests and in clinical studies may reflect the absence of other constituents found in cigarette smoke.

One approach in the treatment of smoking cessation is NRT, which includes over-the-counter treatments such as nicotine gum, patches, nasal spray, and electronic cigarettes. The goal of NRT is to provide nicotine to a smoker without using tobacco, thereby relieving nicotine craving or withdrawal symptoms as the smoker breaks the behavior of cigarette smoking. However, clinical studies show that relapse rates in

smokers who quit with NRT are similar to rates of those who quit without it (Fiore & Jaén, 2008; J. R. Hughes et al., 1999; Ouglas et al., 1999). Here we show that a priming injection of nicotine alone significantly reinstated drug-seeking behavior in animals that had previously self-administered CSE but not in those that had self-administered nicotine. This suggests that cigarette smoke constituents may sensitize brain responses to nicotine, ultimately resulting in more intense craving. This may explain why NRT is not effective as a smoking cessation aid and perhaps should not be used long term.

Nicotinic receptors have been shown to have an important role in mediating both nicotine- and cue-induced reinstatement of nicotine-seeking behavior. For instance, the non-selective nAChR antagonist, mecamylamine, blocks nicotine self-administration and cue-induced reinstatement of nicotine-seeking, as well as nicotine-primed reinstatement of CPP (Biala et al., 2010; Costello et al., 2014; Toll et al., 2012). Furthermore, varenicline, a partial agonist of $\alpha 4\beta 2$ nAChRs and agonist at $\alpha 7$ nAChRs, decreased, and in some cases increased, nicotine-primed reinstatement, while $\alpha 7$ blockade with MLA, but not $\alpha 4\beta 2$ blockade with DH β E, attenuated cue-induced reinstatement of nicotine-seeking in rats (Cippitelli, Wu, et al., 2015; Le Foll et al., 2012.; Li, Li, Pei, Le, & Liu, 2012b; O'Connor et al., 2010). These studies highlight the specific role that different nAChR types have in nicotine- primed and cue- induced reinstatement. Although much work has been done to investigate the involvement of $\alpha 4\beta 2$ and $\alpha 7$ nAChRs in drug- and cue-induced reinstatement, the role of $\alpha 3\beta 4$ nAChRs has not been as thoroughly studied. These receptors are highly expressed in the habenulo-interpenduncular (Hb-IPN) circuit, a tract that is an important mediator of the aversive properties of nicotine, including the withdrawal syndrome following nicotine abstinence (Fowler et al., 2011; Glick et al.,

2006; Grady et al., 2009; Salas et al., 2009a; Stoker et al., 2012; Viswanath et al., 2014). In agreement with published work (Cippitelli et al., 2015b), I now demonstrate that AT-1001 blocks cue- + nicotine-primed reinstatement of nicotine-seeking, emphasizing the importance of $\alpha 3\beta 4$ nAChRs in drug-primed craving.

Since animals that previously self-administered CSE showed enhanced responding to a priming dose of nicotine it seems likely that nAChRs are also involved in this behavior. Confirming this, AT-1001 dose dependently attenuated drug- + cue-primed reinstatement in animals that had self-administered CSE, but to a lesser extent than in animals that had self-administered nicotine. AT-1001 was less potent in attenuating drug- + cue-induced reinstatement of CSE seeking than of nicotine-seeking. This was the case whether the priming drug was CSE or nicotine alone, suggesting that the decreased potency of AT-1001 may be due to an altered functional interaction of $\alpha 3\beta 4$ nAChRs as a result of prior self-administration of CSE, however further investigation is necessary to show this.

In conclusion, I have shown that the inclusion of aqueous cigarette smoke constituents in nicotine reinstatement studies contributes to the increased tendency for reinstatement. The present results suggest that nicotine is the primary constituent in CSE mediating drug-primed reinstatement, and that the inclusion of the aqueous constituents in CSE sensitize animals to the behavioral effects of nicotine- priming and drug-associated cues and this perhaps leads to a decreased effect of $\alpha 3\beta 4$ nAChR blockade of drug + cue induced reinstatement. Nevertheless, $\alpha 3\beta 4$ nAChR functional antagonism dose-dependently blocked reinstatement of both CSE- and nicotine-seeking behavior, confirming a role of this nAChR in cue- + drug-primed reinstatement. In all, these

findings demonstrate the importance of including whole smoke constituents in preclinical models of tobacco dependence. They also suggest that $\alpha 3\beta 4$ nAChR functional antagonism may be a suitable treatment approach to reduce craving during smoking cessation.

Ch. 3

Chronic Exposure to CSE Enhances Withdrawal in Adult and Adolescent Rats

INTRODUCTION

There is much evidence that physical dependence plays an important role in compulsive drug use (NIDA, 2010). Abrupt cessation of tobacco use in dependent individuals results in withdrawal symptoms (al'Absi et al., 2004; O'Dell et al., 2004; Paolini and De Biasi, 2011). In humans, withdrawal from tobacco use leads to somatic symptoms, such as bradycardia, insomnia, and gastrointestinal discomfort, and negative affective symptoms such as irritability, depressed mood, anxiety, and difficulty concentrating (Hughes, 1986; Hughes et al., 1991; Hughes and R., 2007). Both somatic and affective symptoms emerge as quickly as 20 minutes after cessation and can persist for months (CDC, 2014; Gilbert et al., 1995; Hughes et al., 1991; Hughes and R., 2007). However, full recovery from affective disturbances may take years and can often end in relapse (West, Schneiders, Russell, & Feyerabend, 1987).

Although controversial, an emerging body of clinical research shows that human adolescents are especially sensitive to withdrawal, exhibiting symptoms of dependence soon after smoking initiation and before the establishment of daily smoking habits (Dierker and Mermelstein, 2010; DiFranza et al., 2007; Zhan et al., 2012). Since smoking typically begins during adolescence (CDC, 2014), it is important to study withdrawal associated with smoking during the adolescent developmental period in order to fully understand tobacco addiction.

Preclinical studies of withdrawal show that, following chronic nicotine, adult rodents show similar symptoms of withdrawal as humans (Bauzo and Bruijnzeel, 2012; Damaj, 2003; Lin et al., 1999; Malin et al., 1992). This provides an invaluable test with great face validity for understanding the mechanisms involved in tobacco withdrawal symptoms, and also provides a way to test the efficacy of potential smoking cessation agents. Adult rodents show similar symptoms of withdrawal as humans after chronic nicotine treatment. However, there are major discrepancies between the findings of human and animal adolescent withdrawal studies. Whereas human adolescents have an increased sensitivity to the effects of smoking cessation (O'Dell et al., 2004; V. Prokhorov, Karen Suchanek Hudmon et al., 2001; Zhang et al., 2012), rodent adolescents are less sensitive than adults to both somatic and affective nicotine withdrawal effects (Natividad et al., 2010; Laura E. O'Dell et al., 2006a). One possible reason for this discrepancy may be that the animals are exposed to nicotine alone and not to other non-nicotine constituents also found in cigarette smoke. Since withdrawal reflects the imbalance in neurocircuitry that results in the absence of a drug in a dependent individual, an improved method to study withdrawal to smoking in preclinical tests is to study cigarette smoke in its entirety. In fact, studies have shown that chronic exposure to tobacco smoke via inhalational exposure induces both physical and psychic dependence, as compared to non-smoke exposed adult rats (Small et al., 2010). Others have demonstrated that pharmacological inhibition of monoamine oxidase isoforms also modulate nicotine withdrawal (Guillem et al., 2008; Malin et al., 2013). Such inhibition of monoamine oxidase is also induced by non-nicotine ingredients in tobacco smoke (Arib et al., 2010; Bacher et al., 2011; Costello et al., 2014).

CSE is a useful tool to study the collective effects of the thousands of cigarette smoke constituents on withdrawal. Since CSE is a solution, we can control the amount of nicotine that the animal is exposed to each day, whereas a consistent measure of nicotine exposure may not be possible via inhalational exposure. Our previous finding that CSE self-administration sensitized animals to stress-induced reinstatement (Costello et al., 2014) may suggest that CSE sensitizes animals to stress responses and to the negative effects associated with smoking dependence. The present study compares the effects of chronic exposure to CSE and nicotine on both somatic and affective measures of spontaneous and precipitated withdrawal in adolescent and adult male rats.

MATERIALS AND METHODS

Animals

Male Sprague Dawley rats (Charles River Labs, Hollister CA) arrived at P17 with dam or P81 and were housed 2-3 per cage (after weaning at P21 for adolescents) in a humidity and temperature controlled vivarium with a 12-hour light cycle, with lights turned on at 7 a.m. daily. To reduce surgical stress, animals were handled for two days prior to catheterization surgery. Adolescent animals were free-fed while adult animals were food restricted to be kept at a 95% free-feeding weight during the duration of experiments.

Drugs

Nicotine hydrogen tartrate (Sigma, St. Louis, MO) was dissolved in sterile saline and adjusted to pH 7.2-7.4. All nicotine doses were calculated as free base amounts.

CSE was created by bubbling the smoke from commercial cigarettes (Camel unfiltered, R.J. Reynolds Co.) through sterile saline (Costello et al. 2014; Gellner et al., 2016). Mecamylamine HCl (Tocris Bioscience, Bristol, UK) was dissolved in sterile saline with dose calculated as salt weight.

Surgery

Adults and adolescents (P26-28) animals were anesthetized with equithesin (0.0035ml/g body weight) and implanted with indwelling jugular vein catheters based on previously published methods (Belluzzi et al, 2005). Catheters were kept patent with daily flushing with a heparinized saline solution (1 ml of 1000 units/mL heparin into 30 ml bacteriostatic saline).

Dependence treatment

Following recovery from surgery, rats received intravenous injections of saline, nicotine or CSE in an operant chamber programmed to deliver one injection per minute for 15 minutes, to yield a total of 0.5 mg/kg nicotine (free base) or CSE nicotine content per session. Rats received three daily sessions (9am, 12pm, 3pm) totaling 1.5mg/kg/day of nicotine content for 10 consecutive days.

Spontaneous somatic withdrawal

For spontaneous somatic withdrawal, rats underwent withdrawal scoring before surgery and drug treatment began, and 1, 4, 18 and 48 hrs after the last drug injection. Somatic symptoms were assessed for 30 min following 30 min habituation to the open

field chamber (17" x 17" x 12") (Med Associates, St. Albans, VT). An observer blind to drug groups scored the following symptoms: body shakes, cheek tremors, eye blinks, genital licks, gasps, head shakes, ptosis, teeth chattering, yawns, and writhes (Malin et al., 1992). Withdrawal was defined as a significant increase in total withdrawal symptoms as compared to the saline group at the same time point. Catheter patency was verified by rapid anesthesia following infusion of 0.1ml of propofol (Abbott Laboratories, Chicago, IL) after scoring the 4 hr withdrawal time point. Animals without patent catheters were excluded from analysis.

Precipitated somatic withdrawal

To investigate nAChR involvement in CSE withdrawal, a separate group of animals received an injection of saline or mecamylamine (1 mg/kg; s.c.), a non-selective nAChR antagonist, immediately following the last drug infusion, and were placed in the open field chamber and scored for somatic withdrawal symptoms for 60 min. Withdrawal was defined as a significant increase in total withdrawal symptoms as compared to the vehicle treated group. Catheter patency was verified for rapid anesthesia by infusing 0.1ml of propofol (Abbott Laboratories, Chicago, IL) immediately following the test. Animals without patent catheters were excluded from analysis.

Spontaneous affective withdrawal

Spontaneous affective withdrawal was measured 18 hrs following the last drug infusion using the light-dark box test for anxiety like behavior. Animals were isolated in a plexi glass cage (16" x 16" x 12") in the behavior testing room for 10 min, then were

placed in the dark side of a light-dark box (17" x 8.5" x 12" each side) (Med Associates, St. Albans, VT) and the time spent in the light versus dark chambers was recorded for 5 min (O'Dell et al. 2015). Anxiety-like behavior was defined as an increase in the time spent in the dark box as compared to the saline group. The same group of animals were tested for anxiety-like behavior using center time analysis 30 days following the last drug infusion. Animals were isolated in a plexi glass cage in the behavior testing room for 10 min, then were placed in the center of an open-field chamber ((2.5", 2.5") to (14", 14")) and the time spent in the center versus the periphery was recorded for 5 min. Anxiety-like behavior I was defined as a decrease in the time spent in the center of the open field chamber as compared to the saline group.

Precipitated affective withdrawal

To measure precipitated affective withdrawal, animals were injected with saline or mecamylamine (1 mg/kg; s.c.) and isolated in a plexi glass cage for 20 min (Pellow et al., 1985). After isolation, and a 5 min room habituation, the rats were placed in the dark side of a light-dark box (Med Associates, CA) and the time spent in the dark versus light chambers was recorded for 5 min. Anxiety-like behavior was defined as an increase in the time spent in the dark compartment as compared to vehicle treated groups.

Data analysis

Age differences in mean total spontaneous somatic withdrawal symptoms following chronic nicotine or CSE treatment were analyzed with a 3-way ANOVA for Age x Drug x Time, with repeated measures on Time. Significant main effects were

analyzed further with ANOVAs and Bonferroni-corrected paired or unpaired t-tests, where appropriate. For spontaneous affective withdrawal, the % time spent in the dark side and the % time spent in the center were analyzed with a 2-way ANOVA for Age x Drug. Significant main effects were analyzed further with Bonferroni-corrected or unpaired t-tests, where appropriate. For precipitated withdrawal, differences in mean total precipitated somatic withdrawal symptoms and % time spent in the dark side were analyzed with a 3-way ANOVA for Age x Drug x Antagonist dose. Significant main effects were analyzed further with ANOVAs and unpaired t-tests.

RESULTS

Spontaneous somatic withdrawal

3-way ANOVA revealed overall effects of Drug ($F_{2,38} = 18.607$, $p = 0.000$), Time ($F_{4,152} = 11.478$, $p = 0.000$), a Time x Drug interaction ($F_{8,152} = 3.982$), and a Time x Age interaction ($F_{4,152} = 2.657$, $p = 0.035$). Age differences were observed in the baseline scoring for all groups (saline $p = 0.004$, nicotine $p = 0.000$, CSE $p = 0.005$), and at 18 hrs for the saline group ($p = 0.014$).

Adult rats treated with CSE exhibited significant spontaneous somatic symptoms at an earlier time point than those treated with nicotine (Fig. 3.1A). Overall ANOVA showed significant effects of Drug ($F_{2,23} = 17.112$; $p = 0.000$), and a significant Time x Drug interaction ($F_{8,92} = 3.209$; $p = 0.003$). CSE withdrawal, as defined as a significant difference from saline-treated animals, was observed 4 hrs after the last CSE infusion ($p < 0.001$), and was significantly greater than that of animals treated with nicotine alone (p

= 0.028). In contrast, significant nicotine withdrawal was not observed until 48 hrs after the last drug infusion ($p = 0.007$), although a trend was seen at 18 hrs ($p = 0.063$).

Cessation of chronic treatment with CSE, but not nicotine, resulted in somatic withdrawal in adolescent rats (Fig. 3.1B). Overall ANOVA showed significant Drug ($F_{2,15} = 7.493$; $p = 0.006$) and Time effects ($F_{4,60} = 3.571$; $p = 0.011$), and a significant Time x Drug interaction ($F_{8,60} = 3.065$; $p = 0.006$). As has been reported previously (O'Dell et al., 2004, Shram et al., 2008), adolescent rats did not show a significant increase of somatic withdrawal symptoms after a moderate dose/schedule of chronic nicotine treatment at any time point. At 18 hrs post-treatment, animals treated with CSE showed significantly higher somatic withdrawal symptoms than those treated with saline ($p = 0.02$) or nicotine ($p = 0.025$).

Precipitated somatic withdrawal

Both adolescents and adults treated chronically with CSE showed higher precipitated somatic withdrawal than animals treated with nicotine (Fig. 3.2). Overall ANOVA showed significant Drug ($F_{1,74} = 16.819$, $p = 0.000$), and Pretreatment effects ($F_{1,74} = 123.144$, $p = 0.000$), with a significant Drug x Pretreatment interaction ($F_{1,74} = 14.871$, $p = 0.000$). No significant age effects were observed. Adolescent and adult animals treated with both CSE and nicotine showed an increase in somatic withdrawal signs after injection with mecamylamine (1 mg/kg; s.c) ($p = 0.000$). However, precipitated withdrawal symptoms were significantly higher in animals chronically treated with CSE than those treated with nicotine ($p = 0.000$)

Spontaneous affective withdrawal

Animals treated with CSE showed higher spontaneous affective withdrawal symptoms than those treated with nicotine at 18 hrs after the last drug treatment (Figure 3.3). There was a significant effect of Drug ($F_{1,51} = 18.281$; $p = 0.000$) but not of Age nor an Age x Drug interaction. Chronic CSE treatment resulted in a significant increase in the time adolescents and adults spent in the dark side compared to chronic treatment with saline ($p = 0.000$) or nicotine ($p = 0.001$). There were no overall Drug or Age effects in total ambulatory counts, showing that the difference observed in the time spent in the dark side was not due to locomotor effects (Figure 3.3).

Thirty days after cessation of drug treatments, there were still significant anxiety-like behaviors, as measured by time spent in an open field (Figure 3.4). At this time point, there was an overall effect of Drug ($F_{2,48} = 5.724$; $p = 0.000$) with a trending Age effect ($F_{1,48} = 3.876$; $p = 0.055$) and Age x Drug interaction ($F_{2,48} = 3.171$; $p = 0.051$). Together, all animals treated with CSE showed less time spent in the center of the box than those treated with saline or nicotine ($p = 0.000$). When split by age, adults showed an overall effect of Drug ($F_{2,22} = 23.021$; $p = 0.01$), where animals treated with CSE spent less time in the center of the box than those treated with nicotine ($p = 0.009$). Adolescents showed an overall Drug effect ($F_{2,26} = 20.694$; $p = 0.000$), where those treated with CSE spent less time in the center of the box than saline- or nicotine-treated animals ($p = 0.000$). Furthermore, adolescents treated with CSE spent less time in the center of the box than adults treated with CSE ($p = 0.032$), showing that adolescents are more susceptible to long-term anxiogenic effects of chronic CSE treatment than adults. The differences

observed in the time spent in the dark side are not due to locomotor effects, as demonstrated by no overall Drug or Age effects on total ambulatory counts (Figure 3.4).

Precipitated affective withdrawal

Mecamylamine did not precipitate affective withdrawal in rats treated with CSE or nicotine (Fig. 3.5). There were no significant effects of Drug, Age, or Mecamylamine dose, in the light-dark box test. Total ambulatory counts showed a significant effect of Age ($F_{1,55} = 9.139$; $p = 0.004$), with adolescents treated with CSE moving less than adults ($p < 0.05$) (Figure 3.5).

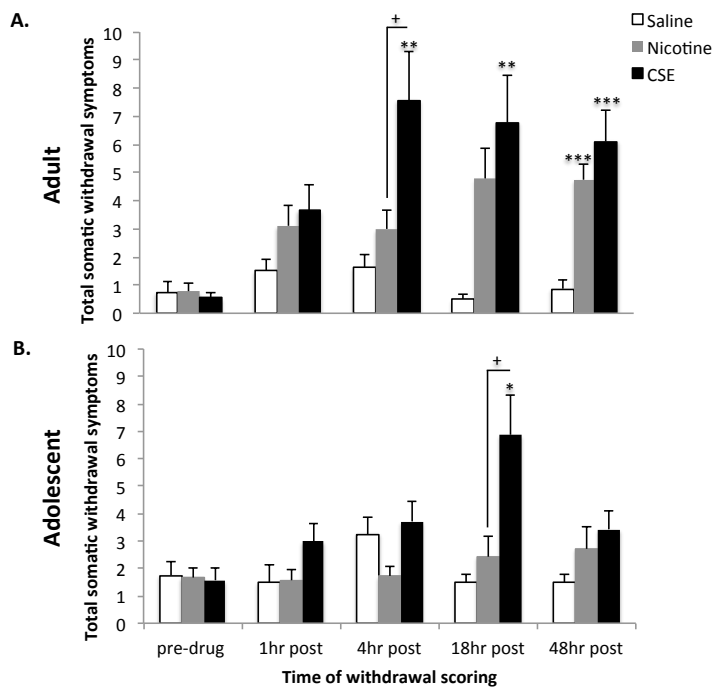


Figure 3.1. Spontaneous somatic withdrawal in adult and adolescent rats **A.** Withdrawal from CSE emerges sooner and is more severe than from nicotine alone in adult rats. **B.** Adolescent rats withdraw after cessation from chronic CSE treatment but not chronic nicotine treatment. Animals were scored for withdrawal abstinence signs at various time points after last drug treatment. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$ vs. saline; + = $p \leq 0.05$ vs. nicotine. n adults = 8-9, n adolescents = 4-7 per group.

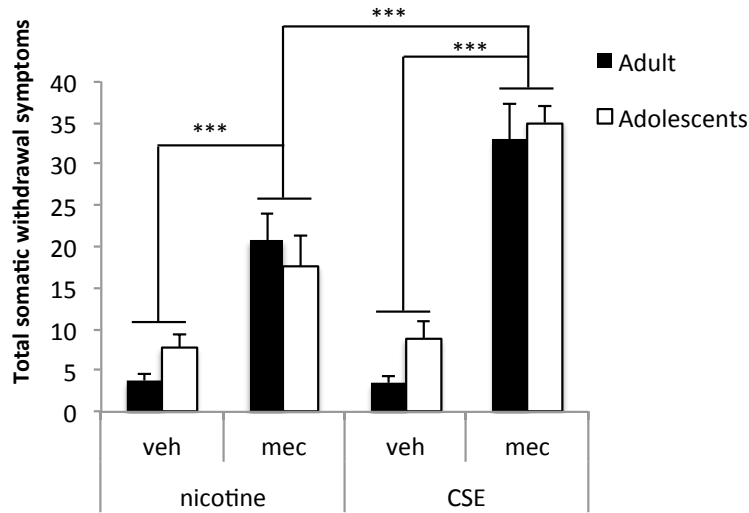


Figure 3.2. Precipitated somatic withdrawal in rats treated with CSE is greater than rats treated with nicotine alone. Animals were given vehicle or mecamylamine 1 mg/kg; s.c.) following drug treatment and scored for somatic withdrawal for 60 min. *** = $p \leq 0.001$. n = 6-11 per group.

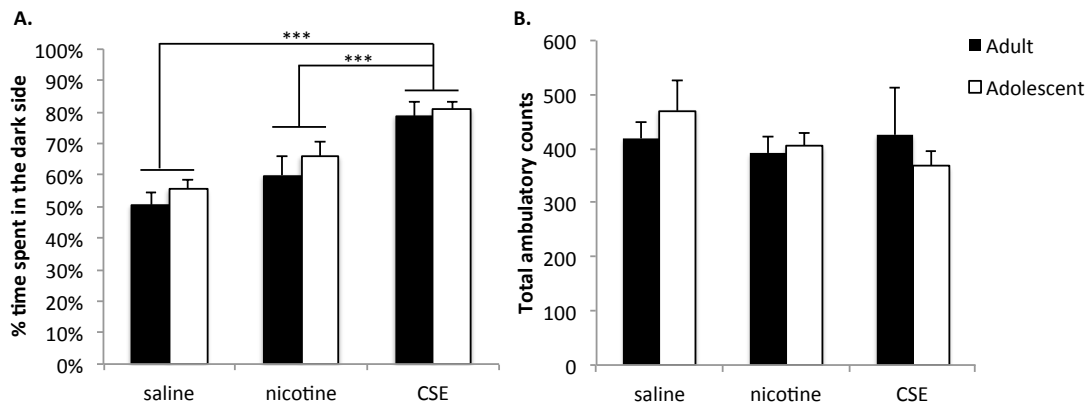


Figure 3.3. Drug, but not age, differences in anxiety-like behavior in a light-dark box test at 18 hrs post drug treatment. A. The time spent in the dark side of the light-dark box was recorded for 5min B. Total ambulatory counts were recorded as a measure of locomotion. *** = $p \leq 0.001$. n = 8-11 per group.

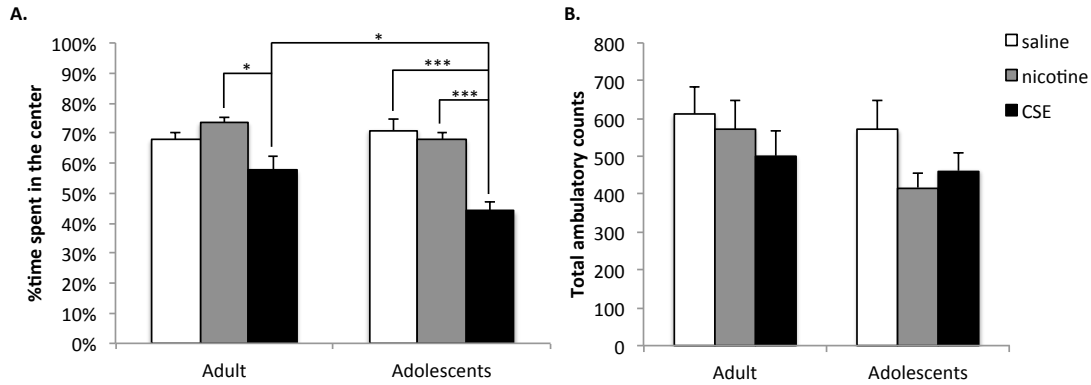


Figure 3.4. Age and drug differences in anxiety-like behavior in an open field at 30 days post treatment. A. The time spent in the center of the open field box was recorded for 5min **B.** Total ambulatory counts were recorded as a measure of locomotion. * = $p \leq 0.05$; *** = $p \leq 0.001$. n = 8-11 per group.

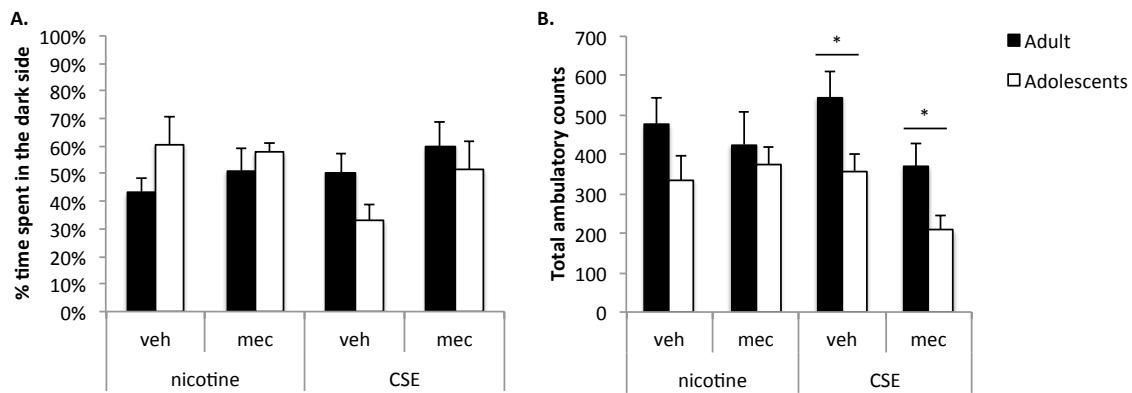


Figure 3.5 Rats treated with CSE or nicotine show no differences in precipitated affective withdrawal. A. The time spent in the dark side of the light-dark box test was recorded for 5min following a saline or mecamylamine injection (1 mg/kg; s.c) **B.** Total ambulatory counts were recorded as a measure of locomotion. * = $p \leq 0.05$ adult vs. adolescent. n = 6-9 per group.

DISCUSSION

The current study has demonstrated that the non-nicotinic constituents of CSE enhance spontaneous somatic and affective withdrawal in adult and adolescent rats as compared to chronic treatment with nicotine alone. Since the nicotine content across drug groups was equal, this finding shows that the non-nicotinic constituents in CSE contribute to neuroadaptations that occur during the formation of dependence that result

in a greater withdrawal syndrome upon cessation. Animals treated with CSE showed higher mecamylamine-precipitated somatic withdrawal than animals treated with nicotine alone, suggesting that cigarette smoke constituents may enhance somatic withdrawal via a nAChR-based mechanism. Whereas CSE treatment increased affective withdrawal as compared to treatment with nicotine alone, the role of nAChRs is less clear, since mecamylamine did not precipitate withdrawal following either CSE or nicotine treatment.

The study of tobacco use with preclinical models has been challenging as it is difficult to find an appropriate method to treat animals in a way that best mimics human smoking. Although studies with smoke inhalation have demonstrated withdrawal after chronic treatment in adult and adolescent rats, these models best mimic second hand smoke (Ponzoni et al., 2015; Small et al., 2010). Another common way to chronically treat animals with nicotine is with an osmotic pump which maintains constant infusions over a chronic period (Damaj, 2003; Malin et al., 2013; O'Dell et al., 2004; Shram et al., 2008). This method does not model the daily perturbations of tobacco use in smokers, however. Nor does it permit alterations in the level of drug delivery as a developing animal grows. The treatment paradigm in the present study was via passive intravenous infusions and not a minipump, because the tar content in cigarette smoke extract would eventually clog the filter in the pump. This approach also allowed daily preparation of CSE, which has non-nicotinic constituents of unknown stability. A further advantage of this intravenous administration approach is that it allows easy control of the dose of nicotine that animals receive daily, which is particularly important in adolescent animals that are experiencing a rapid growth spurt. Thus, in contrast to earlier studies (Laura E. O'Dell et al., 2006b; Shram et al., 2008; Carrie E Wilmouth and Spear, 2006), the current

infusion methodology allows direct comparison of the effects of equivalent chronic drug doses in adolescent and adult rats. However, given the novelty of the methodological approach, it does not fully allow comparison with the findings of earlier studies.

Prior studies have shown that nAChRs have a prominent role in withdrawal. Central nAChRs have been implicated in both somatic and affective symptoms of withdrawal (Watkins et al., 2000). Null mutation of $\beta 2$ nAChR subunits in mice attenuates spontaneous and precipitated withdrawal-induced anhedonia but not somatic withdrawal signs (De Biasi and Salas, 2008; Stoker et al., 2012). Whereas $\beta 4$ knock out mice do not display somatic signs of nicotine withdrawal, nicotine-withdrawn $\alpha 7$ KO mice show increases in anxiety-like behavior but did not withdrawal-induced hyperalgesia or decreases in locomotor activity (Grabus, Martin, & Damaj, 2005; K J Jackson, Muldoon, De Biasi, & Damaj, 2014; Salas, Pieri, & De Biasi, 2004). Chronic nicotine has been shown to enhance nAChR binding of $\alpha 7$ and $\alpha 4\beta 2$ receptors, which may suggest a mechanism as to how nAChRs contribute to the withdrawal syndrome (Doura et al., 2008; Slotkin et al., 2004; Trauth et al., 1999).

Adolescent rodents have been shown previously to be less sensitive than adults to the effects of nicotine withdrawal (Laura E. O'Dell et al., 2006a), which has been attributed to developmental differences in nAChR upregulation (Doura, et al., 2008). However, this finding is in contrast to human studies which suggest that adolescents experience greater withdrawal than adults (Dierker and Mermelstein, 2010; DiFranza et al., 2007; Zhan et al., 2012). It is therefore of particular importance to note that both somatic and affective withdrawal were observed in adolescent rats that were chronically treated with CSE. As with adults, withdrawal symptoms in adolescent rats were more

pronounced following chronic treatment with CSE than with nicotine. Although spontaneous withdrawal from CSE resulted in somatic symptoms in adolescents of similar intensity to those seen in the adult, they were of shorter duration. In contrast, the affective, anxiety-like symptoms seen in adolescents following chronic CSE treatment increased with time and were greater at 30 days post-drug treatment than at 18 hrs. Thus, the withdrawal syndrome that adolescents experience following chronic CSE exposure may be different from that in adults, with greater affective than somatic symptoms and a different time course.

Although chronic treatment with CSE, but not nicotine, resulted in spontaneous affective symptoms in adult and adolescent rats, mecamylamine did not precipitate affective withdrawal in any treatment group. This is in contrast to what was observed for somatic withdrawal symptoms. Others have demonstrated that spontaneous and mecamylamine-induced withdrawal may not yield the same behavioral effects when using the same test. For instance, mecamylamine-precipitated withdrawal following chronic nicotine treatment is accompanied by an increase in the time spent in the periphery of the open field, an effect which was not seen in spontaneous withdrawal (Treit and Fundytus, 1988; Tzavara et al., 2002). The lack of precipitated affective withdrawal may be due to the type of test used, as a light-dark box test may not be sensitive enough to pick up differences in anxiety-like behavior induced by mecamylamine. However, this may also suggest differences in the neuronal mechanisms responsible for the increase in anxiety behavior observed during either precipitated or spontaneous withdrawal. For instance, post-translational effects may need to take place upon cessation of drug treatment in order to observe an effect. It has been reported that

nicotine treatment during adolescence, but not adulthood, results in a negative affective state that is time dependent as it was not observed until 30 days after exposure (Iñiguez et al., 2008). This delay in emergence of affective symptoms has been linked to the mesolimbic expression of stress-related genes induced by nicotine exposure, specifically in adolescent rats (Guzman et al., 2016). The current findings are in agreement with this, and show that treatment with CSE induces a persistent increase in anxiety behavior in adolescents, which is greater than that seen in adults or following chronic nicotine treatment. This may suggest that there is a greater impact of cigarette smoke constituents on stress-related gene expression, but this requires further investigation.

Together, these findings show the important contribution of the non-nicotine cigarette smoke constituents in dependence and withdrawal. CSE may serve as a better tool to study dependence in animals.

Ch. 4

Chronic Exposure to CSE Upregulates Nicotinic Receptor Binding in Adult and Adolescent Rats

INTRODUCTION

Smokers show an “upregulation” of nAChRs that is observed via increases in radioligand binding (Marks, 1983; Schwartz and Kellar, 1983). Radioligand binding studies in postmortem brain (Perry et al., 1999) and MRI studies show up to a 30% increase in nAChR binding (Brody et al., 2013) in heavy smokers. After chronic nicotine treatment, rodents show up to a 110% increase in radioligand binding which has been shown to be dependent on nAChR type, brain area, nicotine dose and treatment paradigm (Perry et al., 2002). Age differences are also observed in this effect (Counotte et al., 2012; Doura et al., 2008; Trauth et al., 1999). Upregulation of $\alpha 4\beta 2$ and $\alpha 7$ nAChRs after chronic nicotine is seen in many brain areas in adult but is more limited in adolescents (Doura et al., 2008). Thus, this resistance to receptor upregulation, may explain why adolescent rodents display less nicotine withdrawal (Badanich and Kirsteina, 2004).

In my previous findings (Ch. 3) adolescents and adult rats showed a robust somatic withdrawal after chronic CSE treatment that was higher than after chronic nicotine treatment. Precipitated somatic withdrawal with mecamylamine, a non-selective nAChR antagonist, was also higher in adult and adolescent animals treated with CSE than nicotine. Furthermore, CSE self-administration enhanced drug craving in reinstatement tests (Ch. 2). It has been proposed that increased expression nAChRs in the brain results in craving and relapse during abstinence (Picciotto et al., 2008; Schwartz and Kellar,

1983). It is therefore possible that the enhanced withdrawal and craving after cessation of CSE treatment results from a change in nAChR pharmacology.

In the present study I have therefore analyzed differences in receptor binding levels after chronic exposure to CSE or nicotine. I hypothesize that the change in nAChR binding after chronic CSE treatment will be greater in both adult and adolescent rats than in nicotine- or saline-treated controls.

MATERIALS AND METHODS

Animals

Male Sprague Dawley rats (Charles River Labs, Hollister CA) arrived at P17 with dam, or at P81, and were housed 2 per cage (after weaning at P21 for adolescents). Animals were kept at a 95% free-feeding weight during the duration of experiments.

Drugs

Nicotine hydrogen tartrate (Sigma, St. Louis, MO) was dissolved in sterile saline and adjusted to pH 7.2-7.4. All nicotine doses were calculated as free base amounts. CSE was created by bubbling the smoke from commercial cigarettes (Camel unfiltered, R.J. Reynolds Co.) through sterile saline (Gellner et al., 2016; Costello et al., 2014).

Surgery

Adults and adolescents (P26-28) animals were anesthetized with equithesin (0.0035ml/g body weight) and implanted with indwelling jugular vein catheters based on previously published methods (Belluzi et al, 2005) and further explained in Ch. 2.

Dependence Induction

Following recovery from surgery, rats received intravenous injections of saline, nicotine or CSE in an operant chamber programmed to deliver one injection per minute for 15 minutes, to yield a total of 0.5 mg/kg nicotine (free base) or CSE nicotine content per session. Rats received three daily sessions (9am, 12pm, 3pm) totaling 1.5mg/kg/day of nicotine content for 10 consecutive days. Here we use an intermittent paradigm of exposure instead of the more commonly used osmotic pump to ensure stability of the cigarette smoke constituents. Additionally, by preparing the drug solution daily we can compensate for adolescent animals' growth.

Autoradiography

Rat brains were extracted immediately after the last drug infusion and flash-frozen in 2-methylbutane at -20°C for 30 secs. Twenty- μ m sections were cut in a cryostat and thaw-mounted onto 4°C positively charged slides. Mounted slides were dried and stored at -20°C with desiccant until processing the next day. Receptor binding was measured in brains using 125 I-epibatidine or 125 I- α -bungarotoxin (Perkin-Elmer, Waltham MA). For 125 I-epibatidine, slides were removed from the freezer and allowed to thaw, then pre-incubated for 10 min in room temperature buffer (50 mM Tris, 120 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, 1 mM MgCl₂, pH 7.4). Binding conditions were varied to selectively label different nAChR types (Perry et al., 2002; Costello et al., 2014). In the α 4 β 2 nAChR binding condition, slides were incubated with 0.08 nM 125 I-epibatidine. Since 125 I-epibatidine also has affinity for other nAChR types, α 4 β 2 nAChR binding was analyzed in brain areas shown to contain at least 85% expression of α 4 β 2

(Perry, et al. 2002). For $\alpha 3\beta 4$ nAChRs, the binding conditions were the same except that 200 nM cytisine (Sigma, St. Louis, MO) was added to the incubation solution to block binding to $\alpha 4\beta 2$ nAChRs. In both conditions, non-specific binding was defined in the presence of 300 μ M nicotine. For ^{125}I - α -bungarotoxin binding, similar conditions were used except the buffer was 50 mM Tris HCl with 120 mM NaCl at pH 7.4. Slides were pre-incubated for 15 min in room temperature buffer, then incubated for 2 hrs with 5 nM ^{125}I - α -bungarotoxin (Perkin-Elmer, Waltham MA). Nonspecific binding was defined in the presence of 10 μ M MLA (Ospina et al., 1998; Ward, et al., 1990). All slides were then washed twice for 10 min in ice-cold buffer, dipped briefly in ice-cold water and blown dry. The dried slides were placed in light-tight cassettes with ^{14}C standards of known radioactivity and exposed to Kodak BioMax MR film for 6-18 hrs for the ^{125}I -epibatidine treated slides or 30 hrs for the ^{125}I - α -bungarotoxin treated slides. Autoradiograms were quantified using an MCID computer-based imaging system (Imaging Research) based on the standards exposed with the slides. Non-specific binding in an adjacent section were subtracted from the total binding in the equivalent anatomical section to calculate specific binding.

Brain areas were chosen based on an *a priori* hypothesis that they might be relevant in negative emotional and other aversive states associated with nicotine dependence, with the focus on areas that contained high populations of the specific nAChR type being studied, according to previous reports (Doura et al., 2008; Perry, et al. 2002). These areas included subregions of the striatum, the limbic system, and the medial habenula and interpenduncular nucleus circuit. Both $\beta 2^*$ and $\alpha 7$ nAChR subunits are highly expressed throughout the brain, including the striatum and limbic system (Doura

et al., 2008; Klink et al., 2001; Perry et al., 2002; Wada et al., 1989). For $\alpha 4\beta 2$ nAChRs, binding was analyzed in the nucleus accumbens core and shell (AcbC, AcbSh), cingulate cortex (Cg cortex), caudate-putamen (CPu), bed nucleus of the stria terminalis (BNST), substantia nigra (SN), and raphe magnus (MnR). For both $\alpha 4\beta 2$ and $\alpha 7$ nAChRs, binding was analyzed in amygdala nuclei, including basolateral (BLA), central (CeA), and medial (MeA), and in lateral hypothalamus (LH). $\beta 4^*$ nAChRs are highly expressed in the habenula-interpeduncular pathway (Hb-IPN), with no mRNA encoding in the LHb by in situ hybridization techniques (Gotti et al., 2009; Grady et al., 2009; Hernandez et al., 2004; Quik et al., 2000). Codistribution of the $\beta 4$ nAChR subunit with the $\alpha 3$ nAChR subunit has been shown in abundance (Winzer-Serhan and Leslie, 1997). Thus for $\alpha 3\beta 4$, binding was analyzed in the medial habenula (MHb), and interpeduncular nucleus (IPN). Some areas highly involved in nicotine dependence or stress responses were excluded from analysis due to limitations in analytical techniques. Brain areas were defined by the “The Rat Atlas” (Paxinos and Watson, 1997).

Data Analysis

Means for regional binding to each nAChR type were determined for animals chronically treated with saline, nicotine, and CSE, and were analyzed with a two-way ANOVA for Age and Drug treatment. Age comparisons were analyzed further with unpaired t-test.

Drug comparisons were analyzed further with Bonferroni-corrected paired t-test.

Receptor upregulation was defined as a significant increase in binding from saline treated controls.

RESULTS

¹²⁵I-epibatidine binding to $\alpha 4\beta 2$ nAChRs

Adolescents show higher levels of ¹²⁵I-epibatidine binding to $\alpha 4\beta 2$ nAChRs than adults in the majority of areas analyzed, in a treatment specific manner (Table 4.1). The following areas showed only significant Age effects (Figure 4.1): AcbSh ($F_{1,54} = 18.317$, $p = 0.000$), AcbC ($F_{1,54} = 16.557$, $p = 0.000$), Cg cortex ($F_{1,45} = 14.877$, $p = 0.000$), CPu ($F_{1,54} = 12.242$, $p = 0.001$), BLA ($F_{1,52} = 18.764$, $p = 0.000$), CeA ($F_{1,52} = 7.820$, $p = 0.007$), LH ($F_{1,53} = 9.204$, $p = 0.004$), MnR ($F_{1,47} = 11.215$, $p = 0.002$).

There was a significant effect of CSE treatment on binding to $\alpha 4\beta 2$ nAChRs in the MeA and a trend in the SN (Table 4.1; Figure 4.2). In the MeA, there was an overall effect of Age ($F_{1,51} = 14.483$, $p = 0.000$), Drug ($F_{2,51} = 7.09$, $p = 0.002$), and an Age x Drug interaction ($F_{2,51} = 3.880$, $p = 0.027$). Adolescents treated with either CSE or nicotine showed higher binding than adults ($p = 0.007$ and 0.015 , respectively). Furthermore, adolescents, but not adults, showed an overall effect of Drug ($F_{2,23} = 5.294$, $p = 0.013$), with those treated with CSE showing significantly higher binding than saline-treated controls ($p = 0.01$). In the SN, there were overall effects of Age ($F_{1,52} = 22.012$, $p = 0.000$) and Drug ($F_{2,52} = 3.489$, $p = 0.038$). Adolescents treated with CSE and nicotine showed significantly higher binding than adults ($p = 0.009$ and 0.005 , respectively). There were no significant differences in binding CSE and nicotine treatment when ages were analyzed separately. When ages were combined, there was a trend towards higher binding after CSE treatment as compared to saline-treated controls ($p = 0.059$) (Table 4.1; Figure 4.2).

There were no overall Age or Drug effects on ¹²⁵I-epibatidine binding to $\alpha 4\beta 2$ nAChRs in the BNST.

¹²⁵I-epibatidine binding to $\alpha 3\beta 4$ nAChRs

CSE treatment resulted in significant upregulation of $\alpha 3\beta 4$ nAChR binding in the MHb and IPN (Table 4.1; Figure 4.3). In the MHb, there was an overall effect of Drug ($F_{2,72} = 3.853$, $p = 0.026$) but not Age. CSE treatment resulted in higher binding than saline-treated controls in both age groups combined ($p = 0.017$). In the IPN, there were overall effects of Age ($F_{1,68} = 5,420$, $p = 0.023$) and Drug ($F_{2,68} = 5.657$, $p = 0.005$). In adolescents there was a significant Drug effect ($F_{2,38} = 3.242$, $p = 0.05$), where CSE treatment resulted in higher binding than controls ($p = 0.045$). There was also a significant Drug effect in adults ($F_{2,30} = 3.644$, $p = 0.038$) where CSE treatment upregulated binding as compared to controls ($p = 0.046$).

¹²⁵I- α -bungarotoxin binding to $\alpha 7$ nAChRs

In general, adolescents show higher levels of ¹²⁵I- α -bungarotoxin binding than adults in the majority of the areas analyzed, in a treatment specific manner (Table 4.1, Figure 4.4, 4.5). In the LH, there was an overall effect of Age ($F_{1,60} = 4.594$, $p = 0.036$), and Drug ($F_{2,60} = 6.951$, $p = 0.002$). In the adolescents there was an overall Drug effect ($F_{2,24} = 5.080$, $p = 0.014$), where CSE treatment resulted in higher binding than nicotine ($p = 0.024$), and controls ($p = 0.048$) (Figure 4.4). Adolescents treated with CSE also showed higher binding than adults ($p = 0.016$).

Whereas age-specific effects of CSE treatment were seen in the LH, those in amygdaloid nuclei were more complex (Figure 4.5). In the BLA, there was an overall effect of Age ($F_{1,65} = 23.748$, $p = 0.000$), and Drug ($F_{2,65} = 4.077$, $p = 0.021$), with adolescents showing higher binding than adults across all treatment groups (CSE $p = 0.012$, nicotine $p = 0.04$, saline $p = 0.002$). Adults showed significant Drug effects ($F_{2,36} = 4.157$, $p = 0.024$), where CSE treatment induced upregulated binding as compared to controls ($p = 0.022$). In the CeA, there was an overall effect of Age ($F_{1,64} = 15.632$, $p = 0.000$), and Drug ($F_{2,64} = 6.563$, $p = 0.003$). Adolescents showed higher binding than adults across all treatments (CSE $p = 0.013$, nicotine $p = 0.047$, saline $p = 0.017$). In adolescents there was also an overall Drug effect ($F_{2,28} = 4.158$, $p = 0.026$), with CSE treatment resulting in higher binding than controls ($p = 0.036$). In the MeA, there was an overall effect of Age ($F_{1,65} = 23.990$, $p = 0.000$), and Drug ($F_{2,65} = 4.516$, $p = 0.015$). Adolescent showed higher binding than adults across all treatments (CSE $p = 0.000$, nicotine $p = 0.015$, saline $p = 0.049$). No individual Drug differences were observed when adults and adolescents were analyzed separately. When ages were combined, animals treated with CSE showed higher binding than those treated with nicotine ($p = 0.018$).

Table 4.1. Binding to nAChRs. AcbSh = accumbens shell, AcbC = accumbens core, Cg cortex = cingulate cortex, CPu = caudate putamen (striatum), BNST= bed nucleus of the stria terminalis, BLA = basolateral amygdala, CeA = central amygdala, MeA = medial amygdala, LH = lateral hypothalamus, SN = substantia nigra, MnR = median raphe nucleus, MHb = medial habenula, IPN = Interpenduncular nucleus. n = 8 – 15 per group. Bolded numbers indicate a significant difference from adult. Gray cells with asterisks (*) indicate a significant difference from saline (* = p ≤ 0.05; asterisks in parenthesis denote a trend of p = 0.059). A cell with a thick border and a cross (+) is a significant difference between nicotine and CSE (+ = p ≤ 0.05).

Brain Region	Adults			Adolescents		
	saline	nicotine	CSE	saline	nicotine	CSE
	Mean ± SEM (DPM/mg)			Mean ± SEM (DPM/mg)		
Binding to α4β2*						
AcbC	600.29 ± 83.66	606.74 ± 79.93	693.20 ± 99.06	987.48 ± 142.14	1049.70 ± 154.48	1022.01 ± 144.49
AcbSh	462.18 ± 69.87	539.78 ± 93.38	557.34 ± 92.49	815.50 ± 113.69	967.65 ± 127.79	992.68 ± 192.03
Cg cortex	630.14 ± 99.76	609.00 ± 92.33	801.94 ± 86.97	968.55 ± 139.17	1290.76 ± 208.38	1376.50 ± 260.37
Cpu	650.30 ± 84.30	755.31 ± 87.54	746.82 ± 90.51	1133.87 ± 198.75	1040.19 ± 128.70	1131.51 ± 208.03
BNST	457.98 ± 75.24	581.90 ± 107.79	648.13 ± 101.26	440.10 ± 60.89	511.96 ± 90.20	574.05 ± 93.76
BLA	396.70 ± 81.47	523.68 ± 104.09	530.09 ± 85.55	748.17 ± 143.61	936.25 ± 87.58	939.56 ± 160.27
CeA	354.75 ± 65.65	345.08 ± 69.14	381.39 ± 60.82	480.08 ± 90.73	619.03 ± 92.72	542.20 ± 112.75
MeA	209.61 ± 28.61	228.66 ± 33.00	267.37 ± 37.49	223.21 ± 37.79	427.78 ± 65.31	607.22 ± 116.04 *
LH	270.46 ± 49.67	324.71 ± 47.94	399.06 ± 55.08	481.05 ± 120.96	548.81 ± 83.84	529.77 ± 104.48
SN	1072.59 ± 135.82	1210.54 ± 113.98	1346.57 ± 180.77 (*)	1576.59 ± 263.77	2398.29 ± 383.15	2562.09 ± 403.44 (*)
MnR	573.15 ± 87.82	653.84 ± 83.99	644.40 ± 130.36	884.58 ± 256.82	1515.39 ± 356.61	1116.36 ± 282.41
Binding to α3β4*						
MHb	33359.70 ± 2964.46	39534.20 ± 2408.91	37233.43 ± 1147.74 *	30175.93 ± 2139.13	36219.86 ± 3910.14	41461.19 ± 2602.97 *
IPN	14680.32 ± 1554.03	19716.59 ± 1474.02	21198.56 ± 1883.04 *	18244.52 ± 1904.76	23363.32 ± 3551.08	27988.87 ± 2682.22 *
Binding to α7						
BLA	923.89 ± 68.35	1005.10 ± 57.80	1164.71 ± 53.10 *	1465.98 ± 148.80	1453.66 ± 140.82	2016.17 ± 333.16
CeA	403.45 ± 57.22	472.64 ± 67.88	585.85 ± 80.19	632.76 ± 69.21	714.98 ± 96.41	1217.65 ± 235.30 *
MeA	708.94 ± 67.55	681.37 ± 50.98	828.98 ± 47.03 +	1029.09 ± 146.99	941.40 ± 90.27	1292.93 ± 104.02 +
LH	719.16 ± 91.46	631.15 ± 66.14	808.05 ± 48.15	719.16 ± 91.46	631.15 ± 66.14	808.05 ± 48.15 * +

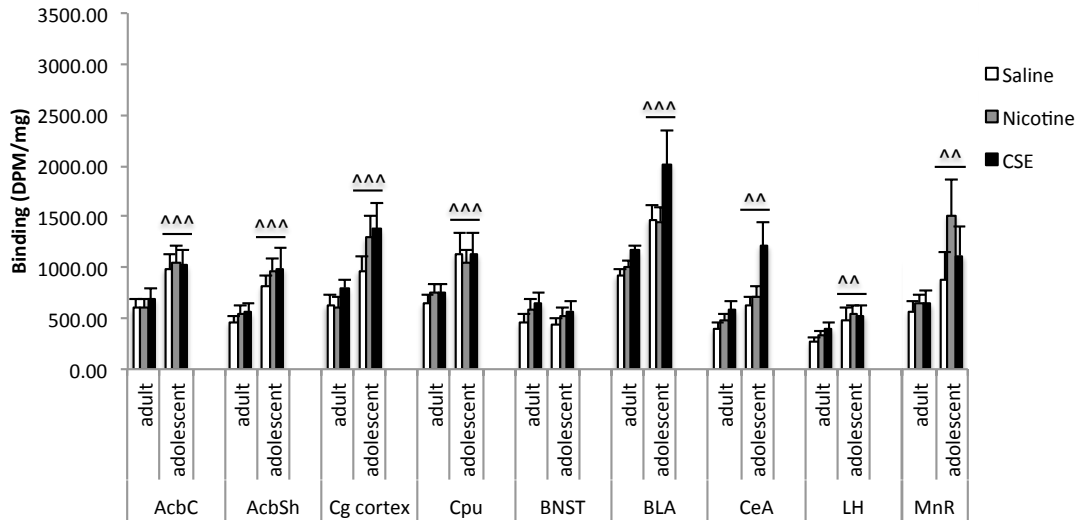


Figure 4.1. ^{125}I -epibatidine binding to $\alpha 4\beta 2$ nAChRs is higher in adolescents than adults. AcbC = accumbens core, AcbSh = accumbens shell, Cg cortex = cingulate cortex, CPu = caudate putamen (striatum), BNST = bed nucleus of the stria terminalis, BLA = basolateral amygdala, CeA = central amygdala, LH = lateral hypothalamus, MnR = median raphe nucleus. $\wedge\wedge = p \leq 0.01$, $\wedge\wedge\wedge = p \leq 0.001$ vs. adult group. $n = 8 - 11$ per group.

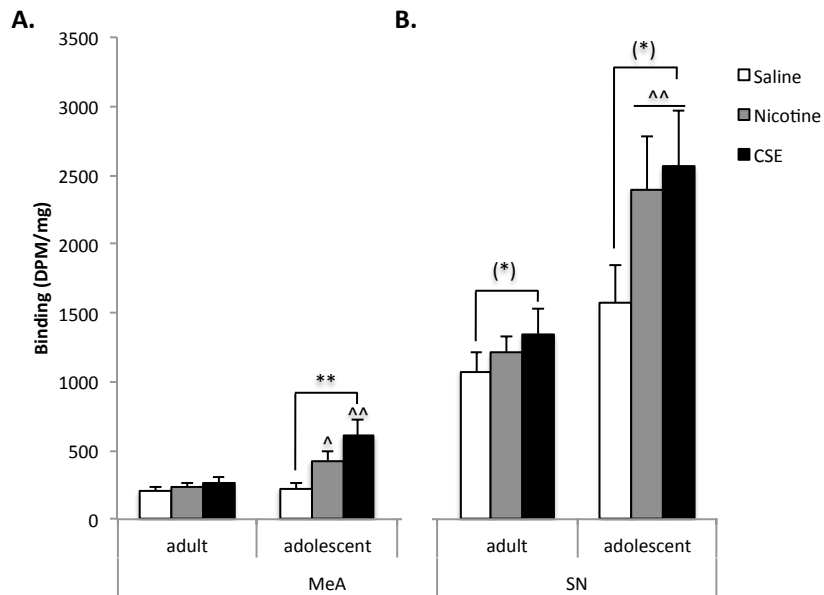


Figure 4.2. ^{125}I -epibatidine binding to $\alpha 4\beta 2$ nAChRs in the medial amygdala and substantia nigra is higher in drug-treated adolescents than adults. **A.** In the MeA, adolescents display a significant CSE-induced upregulation. **B.** In the SN, adult and adolescents show a trend CSE-induced upregulation. MeA = medial amygdala, SN = substantia nigra. $** = p \leq 0.01$; $(*) = p = 0.059$ vs. saline group. $\wedge = p \leq 0.05$, $\wedge\wedge = p \leq 0.01$, $\wedge\wedge\wedge = p \leq 0.001$ vs. adult group. $n = 8 - 11$ per group.

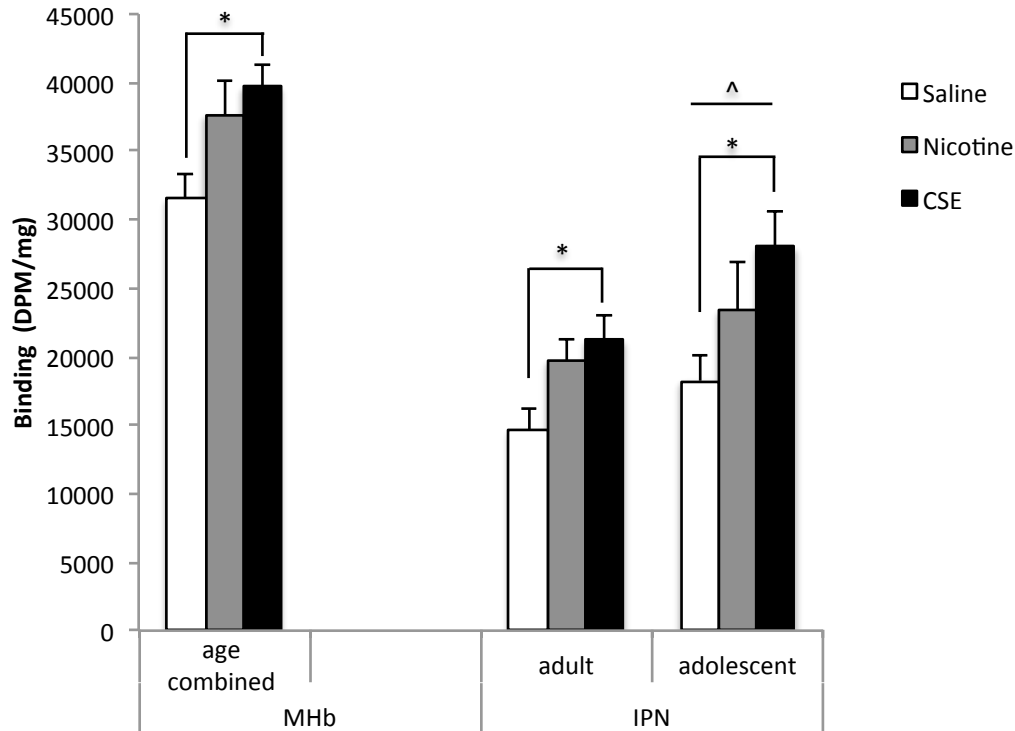


Figure 4.3. CSE-induced upregulation of ¹²⁵I-epibatidine binding to α3β4 nAChRs in the MHb and IPN independent of age, though adolescents show higher overall binding in the IPN. MHb = medial habenula, IPN = Interpeduncular nucleus. * = p ≤ 0.05 vs. saline group. ^ = p ≤ 0.05 vs. adult group. n = 11 – 15 per group.

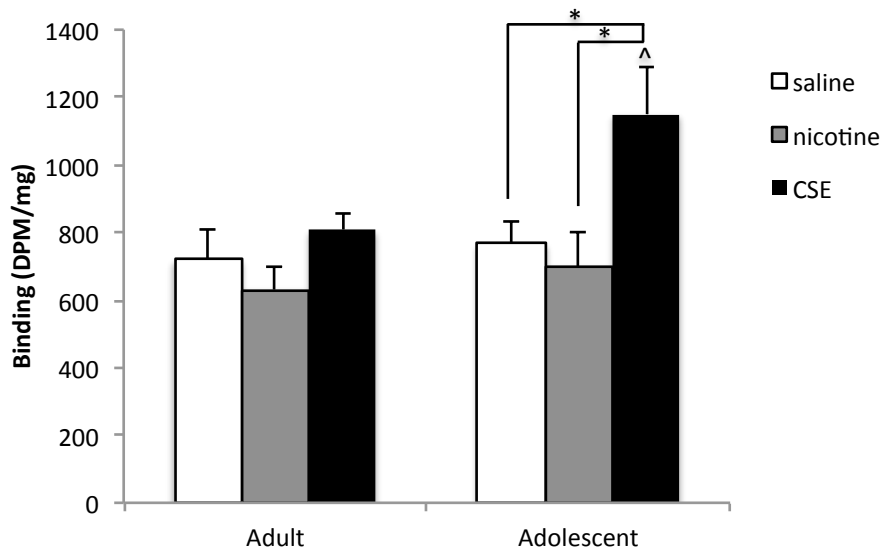


Figure 4.4. ¹²⁵I-α-bungarotoxin binding to α7 nAChRs is increased in the lateral hypothalamus of adolescent rats chronically treated with CSE. * = p ≤ 0.05. ^ = p ≤ 0.05, vs. adult group. n = 8 – 13 per group.

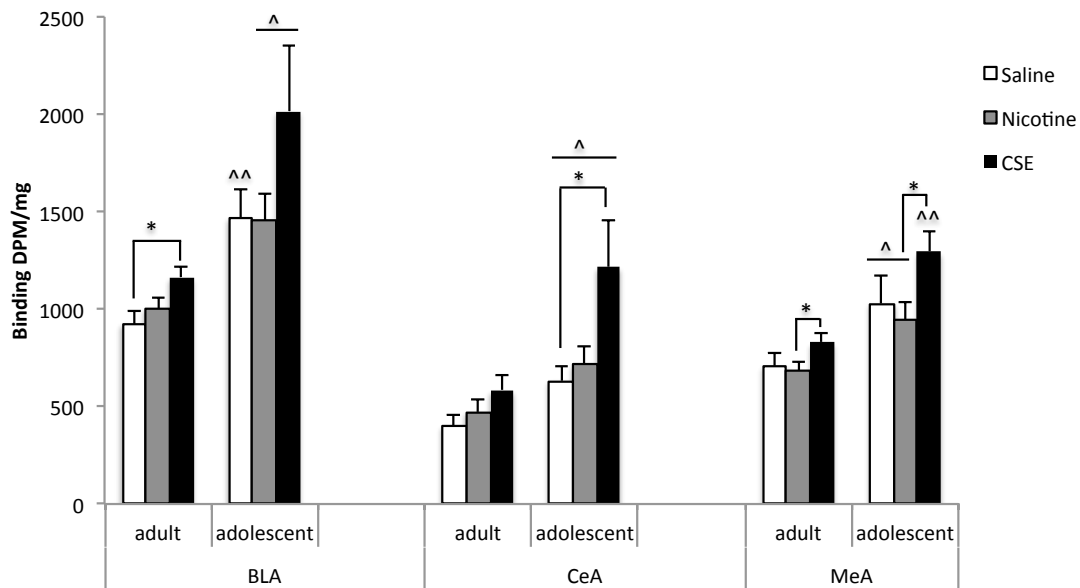


Figure 4.5. ¹²⁵I-α-bungarotoxin binding to α7 nAChRs is increased in amygdala nuclei of rats chronically treated with CSE in an age-dependent manner. BLA = basolateral amygdala, CeA = central amygdala, MeA = medial amygdala * = p ≤ 0.05 vs saline group. ^ = p ≤ 0.05, ^^ = p ≤ 0.01 vs. adult group. n = 9 – 13 per group.

DISCUSSION

The present study is the first to show that cigarette smoke constituents enhance nicotine-induced upregulation of nAChR radioligand binding in both adult and adolescent rodents. In agreement with earlier studies, age differences in radioligand binding were apparent in saline-treated control animals, with adolescents showing higher binding in many regions than their adults counterparts. However, in contrast to earlier findings that show that adolescents demonstrate a limited nAChR upregulation compared to adults (Doura et al., 2008), here adolescents chronically treated with CSE show higher upregulation in many regions than their adult counterparts.

In contrast to other studies, chronic nicotine did not induce significant upregulation of nAChRs in this study. This difference may be due to different methods of drug exposure. Studies of chronic nicotine exposure use osmotic pumps. Here we use

intermittent intravenous injections (for reasons that are discussed in Ch.5). The method of exposure, as well as nicotine dose, has been shown to influence the rate and level of receptor upregulation. For instance, transient exposure of high-dose nicotine seems to favor $\alpha 6\beta 2$ upregulation rather than $\alpha 4\beta 2$ upregulation, which is induced by prolonged exposure of low dose nicotine (Nashmi et al., 2007). Since our animals are receiving high concentration (1.5 mg/kg nicotine content per day) in three intravenous sessions per day for 10 days, it is possible that not all nAChRs are responsive to the effects of nicotine using this schedule of drug exposure. Nevertheless, the inclusion of cigarette smoke constituents has sensitized the receptors to nicotine-induced upregulation using this exposure method.

An important goal of this study was to get a deeper understanding of the neuropharmacological adaptive mechanisms within circuits that mediate the transition from initial tobacco use to dependence and withdrawal. Thus, the focus was to examine nicotine- and CSE- induced upregulation of nAChR binding in brain areas involved in the dysregulation of the positive reinforcing properties of drugs or the recruitment of the negative reinforcing properties of drugs of abuse. In summary, CSE-induced upregulation was observed in the SN, amygdala, LH, MHb and IPN. The anatomical connections between these areas and how they influence the withdrawal syndrome, as well as our findings, are summarized in Figure 4.6 and are discussed further below.

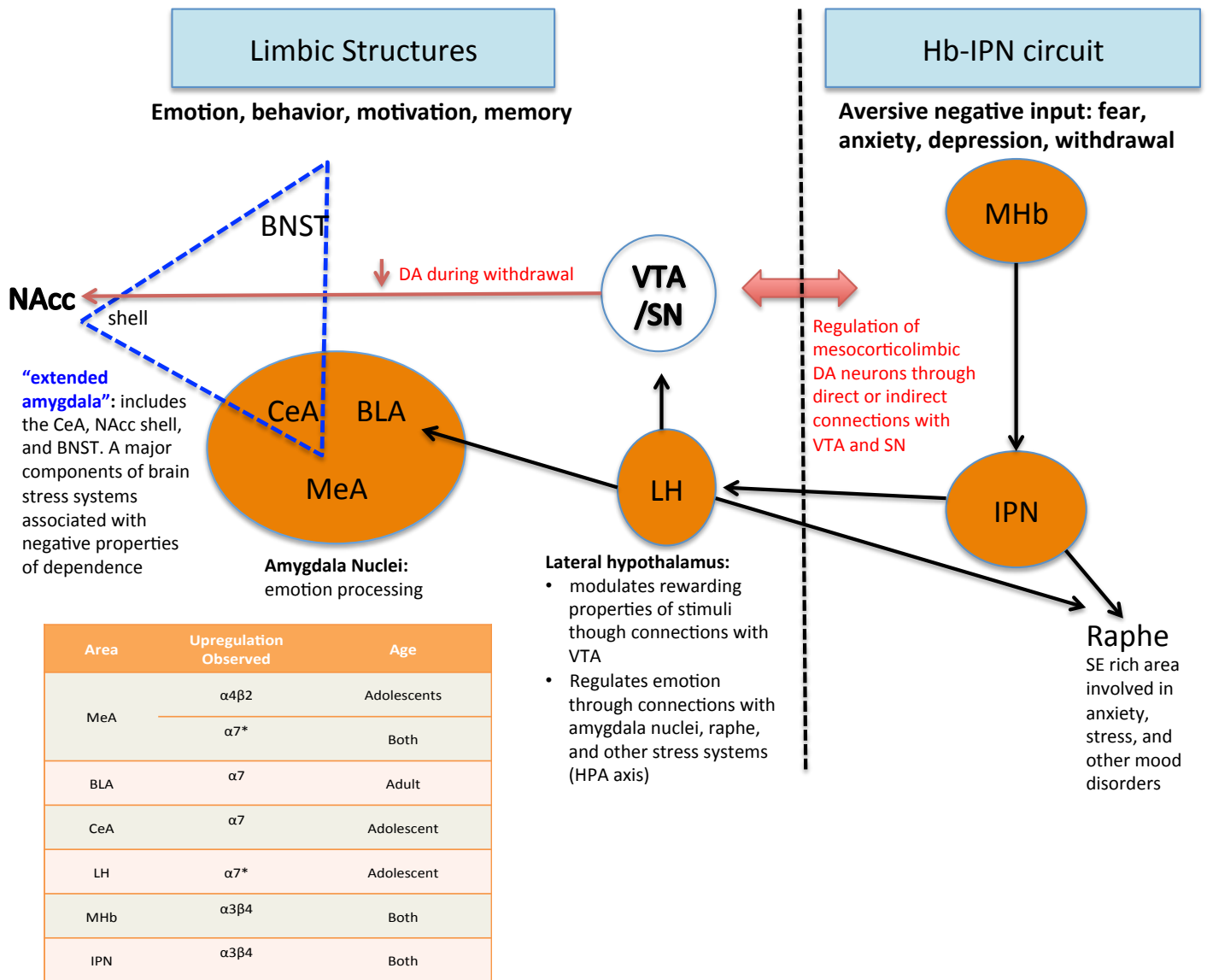


Figure 4.6 Anatomical connections within the limbic system and HB-IPN circuit that mediate withdrawal. Orange circles represent brain areas where nAChR upregulation was observed. The table summarizes the increased binding to nAChRs observed after chronic CSE exposure. The asterisk (*) denotes a significant increase from nicotine treated rats.

The change from positive to negative reinforcing effects of drugs of abuse are mediated by neurotransmitter systems in the striatum either directly or via indirect actions in the ventral tegmental area (VTA) and SN (Koob, 2008; Koob et al., 1993; Koob and Le Moal, 2008; Koob and Volkow, 2010). Methodology limitations did not allow for the analysis of nAChR binding in the VTA; however in the SN, CSE-induced upregulation of

$\alpha 4\beta 2$ nAChRs was observed in both adolescent and adult rats. Upregulation in nAChR binding however was not observed in the NAcc or CPu. Several areas modulate the negative aversive state of nicotine dependence by facilitating DA output from the VTA and SN. These areas include the amygdala, LH, and MB-IPN circuit (Grace et al., 2007; Hildebrand et al., 1998; Kenny and Markou, 2001; Natividad et al., 2010; Zhang et al., 2012). Upregulation was observed in these areas in a subtype and age specific manner:

Adolescents, but not adults, displayed a CSE-induced upregulation of $\alpha 4\beta 2$ nAChRs in the MeA and $\alpha 7$ nAChRs in the CeA and LH. The involvement of these areas in the shift to negative reinforcement and the negative emotional state of withdrawal (Antolin-Fontes et al., 2015; Koob and Volkow, 2010; Narita, 2006) and the important role of $\alpha 4\beta 2$ and $\alpha 7$ nAChRs in affective withdrawal (Maskos et al., 2005; Salas et al., 2004.; Stoker et al., 2012) may explain why CSE-treated adolescents displayed enhanced prolonged affective withdrawal compared to adults (Ch.3).

In the BLA, adults displayed a CSE-induced upregulation of $\alpha 7$ nAChRs. The BLA is involved in craving, a component of withdrawal that often leads to relapse (Koob and Volkow, 2010). Also, $\alpha 7$ nAChRs have proven to be important in mediating drug- and cue-induced reinstatement (Le Foll et al., 2012.; Li et al., 2012b; O'Connor et al., 2010). Thus, a CSE-induced upregulation of $\alpha 7$ nAChRs in the BLA may explain the enhancement in drug- and cue-induced responding in CSE animals (Ch.2).

Analysis of $\alpha 3\beta 4$ nAChRs revealed CSE-induced upregulation in the MHb and IPN in both age groups. $\beta 4^*$ receptors in particular have been shown to have an important role in nicotine withdrawal (Grabus et al., 2005; Grady et al., 2009; Hernandez et al., 2004; Kia J Jackson et al., 2013; Quik et al., 2000; Salas et al., 2004). Thus, an increase

in ligand binding to $\alpha 3\beta 4$ nAChRs in the MHb and IPN may also explain an increased sensitivity to somatic and affective nicotine withdrawal observed in CSE treated adult and adolescent animals.

In summary, chronic treatment of CSE results in an upregulation of $\alpha 4\beta 2$, $\alpha 7$, and $\alpha 3\beta 4$ nAChRs binding, more than chronic nicotine treatment. Overall, nAChR binding was higher in adolescent than adult rats but both age groups were susceptible to upregulation after chronic CSE treatment. However, upregulation was observed in different brain regions based on age. Overall, upregulation was observed in amygdala nuclei, LH, SN, and the MHb- IPN; brain areas critical for mediating the negative aspects of nicotine. The pattern of upregulation coincides with increases in withdrawal and craving observed in animals chronically treated with CSE, suggesting a relationship between receptor upregulation and increased drug dependence potential. In conclusion, these results provide evidence that the cigarette smoke constituents in CSE influence nAChR pharmacology that may be uniquely based on age group.

Chapter 5

General Discussion

Tobacco dependence is a large public health problem remaining one of the leading causes of preventable death in the United States (CDC, 2014). Despite heightened awareness of the detrimental consequences of smoking, over 23% of adults in the United States continue to smoke. Although most smokers express the desire to quit, the great majority will not succeed at doing so (Hughes et al., 1992). Even with the help of treatments for smoking cessation such as nicotine replacement therapy, bupropion or varenicline, no more than 23% of smokers will stay abstinent for a full year (Jorenby et al., 2006; Smith et al., 2008). Although absolutely necessary, more effective smoking cessation therapies have not yet been discovered, in spite of the extensive research and funding gone into the pursuit. Considering that most pharmacotherapies for smoking cessation fail in the clinical stage of development, I believe that in order to develop more efficacious therapies for smoking cessation, preclinical models of tobacco dependence must be improved. To date, most preclinical tests used to assess the efficacy of smoking cessation therapies use nicotine alone. However, I believe this to be an oversimplified model of tobacco dependence as it ignores the thousands of other cigarette smoke constituents that have been shown to influence nicotine addiction (Belluzzi et al., 2005; Costello et al., 2014; Gellner et al., 2016). Furthermore, experimental tests are generally performed on adult animals, although the majority of smokers start using tobacco products during adolescence. Thus, preclinical tests of tobacco dependence can be improved by creating a model of smoking that includes cigarette smoke constituents and

examines both adults and adolescents. By doing so, we can obtain a better understanding of the neural mechanisms involved in tobacco craving and withdrawal, two factors that highly influence relapse in smokers. This, along with finding and validating new targets for smoking cessation, has the potential to greatly increase the efficacy of these treatments and better preserve the health of the general public.

The adolescent stage of development is the most critically impacted by the negative aspects of cigarette smoking. Thus to fully understand smoking addiction, it is imperative to study smoking during the less studied adolescent period. The over-simplicity of using nicotine alone in preclinical tests of smoking can be seen in the effects that nicotine alone has on adolescents. For instance, adolescent rodents do not demonstrate the increased sensitivity to withdrawal following chronic nicotine exposure that teenage smokers experience (Carcoba et al., 2014; Laura E. O'Dell et al., 2006a; V. Prokhorov, Karen Suchanek Hudmon et al., 2001; Zhang et al., 2012). However, adolescent rats exposed to cigarette smoke via passive inhalation exhibited increased anxiety-like behavior during withdrawal (De la Peña et al., 2016). This demonstrates how inclusion of cigarette smoke constituents can influence dependence to tobacco and serves as a more valid preclinical model for smoking.

In an attempt to create a more valid preclinical model of smoking, our lab has created CSE, an aqueous cigarette smoke extract that animals reliably self-administer (Costello et al., 2014, Gellner et al., 2016). We have demonstrated CSE to be a more potent drug than nicotine alone in self-administration tests (Costello et al., 2014). Furthermore, CSE sensitizes animals to stress-induced relapse, as shown on extinction-reinstatement tests. When used to assess the efficacy of a novel pharmacotherapy for smoking cessation, the

reduction of drug intake was less in animals self-administering CSE than nicotine alone. Together these findings establish the improved validity of using CSE instead of nicotine in preclinical tests of tobacco dependence. However, an investigation of the effects of CSE on paradigms of drug- and cue-induced craving and withdrawal must be done in order to obtain a more thorough evaluation of CSE as a preclinical tool to assess therapies for smoking cessation.

The general goal of this dissertation was to investigate the effects of cigarette smoke constituents on preclinical paradigms of drug craving and withdrawal, with the general hypothesis that the cigarette smoke constituents would enhance the addictive potential of nicotine alone. I have compared the effects of nicotine and CSE on drug- and cue-induced relapse, and used this model to validate a new target for smoking cessation treatments. I also compared the effects of nicotine and CSE on spontaneous and precipitated somatic and affective withdrawal in both adult and adolescent rats, which then lead me to explore differences in nAChR binding as a means to investigate CSE- or nicotine-induced changes in nAChR properties.

Role of $\alpha 3\beta 4$ nicotinic acetylcholine receptors in cue- + CSE- and nicotine-primed reinstatement of drug-seeking behavior

A previous study in our lab has shown that CSE is a tool with better face validity than nicotine alone in self-administration and stress-induced reinstatement tests (Costello et al., 2014). However, no tests to date have investigated the effects of CSE self-administration on drug- and cue- induced reinstatement. In preclinical studies, nicotine alone was not potent at inducing reinstatement unless drug-associated cues are present (Chaudhri et al., 2007, 2006; Sorge et al., 2009). Thus, I hypothesized that animals that

had self-administered CSE would reinstate responding with drug priming alone, not requiring the presentation of cues, and thus providing further evidence that tobacco smoke constituents enhance the propensity to relapse as compared to nicotine alone. As hypothesized, my findings demonstrated that unlike animals that self-administered nicotine, animals that self-administered CSE reinstated with a priming dose of CSE- or nicotine- alone. In fact, CSE animals showed significantly higher responding than nicotine animals to a priming dose of nicotine alone or cues alone. This suggested that nicotine is the primary constituent in CSE mediating drug-primed reinstatement, and that the inclusion of the aqueous smoke constituents in CSE causes a behavior sensitization to nicotine- and cue- induced craving.

Since both nicotine- and cue-induced reinstatement have been shown to be mediated by nAChRs (Le Foll et al., 2012.; Li et al., 2012a; O'Connor et al., 2010), I further tested the hypothesis that repeated CSE exposure alters nAChR properties, resulting in a sensitivity to reinstatement. To this end, I wanted to focus on the involvement of $\alpha 3\beta 4$ nAChRs, which have shown promise as a new target for tobacco dependence treatment. Genome-wide association studies have shown that polymorphisms in the gene cluster encoding for the $\alpha 3-\alpha 5-\beta 4$ nAChR subunits are associated with an increased risk for tobacco dependence (Berrettini et al., 2008). However, investigation of $\alpha 3\beta 4$ nAChR involvement in drug-primed reinstatement has been limited. Through a collaboration with Astrea Therapeutics, we have tested the effect of AT-1001, a functional antagonist of $\alpha 3\beta 4$ nAChRs, on CSE and nicotine self-administration, and have found that it was more efficacious in reducing nicotine self-administration (Costello et al., 2014). I thus used AT-1001 to test my hypothesis that $\alpha 3\beta 4$ nAChRs play a role in

cue- + drug-primed reinstatement, and that cigarette smoke constituents will reduce the potency of AT-1001 at blocking reinstatement. Confirming this, AT-1001 dose-dependently attenuated cue- + drug-primed reinstatement in animals that self-administered CSE, but to a lesser extent than in animals that self-administered nicotine alone. The 0.75 mg/kg dose of AT-1001 fully attenuated reinstatement in animals that had previously self-administered nicotine alone, but did not inhibit reinstatement of CSE-seeking behavior regardless of whether CSE or nicotine was used as drug prime. This finding confirms the importance of $\alpha 3\beta 4$ nAChRs in cue- + nicotine-primed craving, and confirms a reduction in AT-1001 potency as a result of prior CSE self-administration, perhaps due to an altered functional interaction of $\alpha 3\beta 4$ nAChRs, though further investigation is necessary to show evidence of this.

Overall, these findings demonstrate the importance of including whole smoke constituents in preclinical models of tobacco dependence. They also suggest that $\alpha 3\beta 4$ nAChR functional antagonism may be a suitable treatment approach to reduce craving for nicotine or cigarettes, though higher dosing may be needed for the latter.

Future Directions

Future studies may clarify if prior CSE self-administration alters $\alpha 3\beta 4$ nAChR pharmacology resulting in a decreased potency of AT-1001. I hypothesize that sub-chronic CSE altered the affinity of the nAChR to nicotine or AT-1001. Previous work from my lab has shown no significant differences in the impact of acute CSE or nicotine treatment on $\alpha 3\beta 4$ nAChR binding (Costello et al., 2014), but I hypothesize that differences will arise after repeated exposure. An ex-vivo competitive binding assay,

following CSE or nicotine self-administration, using ^{125}I -epibatidine + cytisine to selectively target $\alpha 3\beta 4$ nAChRs and either AT-1001 or nicotine to displace binding may be used to test this hypothesis.

Future studies may also investigate the role of other nAChRs, such as $\alpha 4\beta 2$ and $\alpha 7$, in CSE -induced reinstatement. We can achieve this by repeating a dose response for attenuation of CSE- or nicotine-primed reinstatement with different antagonists such as DH β E, a $\alpha 4\beta 2$ nAChR partial agonist, and methyllycaconitine (MLA), a $\alpha 7$ nAChR antagonist.

Future tests could evaluate possible age differences in drug-primed reinstatement in adolescent animals. To date, drug-primed reinstatement has not been investigated in adolescent animals because of the methodological difficulty of using a two-lever operant chamber in this age group. However, our lab has recently optimized the paradigm in adolescent animals using stress-induced reinstatement, making this type of analysis of drug priming possible. Thus, it would be interesting to observe possible age differences, or drug differences, in adolescent susceptibility to drug-primed reinstatement and the efficacy of AT-1001 to attenuate it.

Chronic exposure to CSE enhances withdrawal in adult and adolescent rats

Withdrawal from smoking is a major factor leading to relapse. Withdrawal is characterized by somatic (physical) and affective (psychic) symptoms that can manifest in the first few hours of cessation, and can last for months after a quitting attempt (Gilbert et al., 1995; Hughes et al., 1991; Hughes and R., 2007). Having studied the craving component of withdrawal with my first aim, I then wanted to evaluate other affective and

somatic symptoms of withdrawal after cessation of chronic CSE treatment. Animal models of chronic nicotine treatment have great face validity in adults, but lack it in adolescents. Whereas human adolescents are reported to be more sensitive to withdrawal from tobacco use than adults, adolescent animals have proven to be resistant to the aversive withdrawal effects of chronic nicotine (Natividad et al., 2013; Laura E. O'Dell et al., 2006a; Zhang et al., 2012). I have hypothesized that this lack of validity in adolescent models was due to an absence of cigarette smoke constituents in the current models of tobacco dependence and, thus, chronic treatment with CSE would result in enhanced withdrawal effects in both adolescent and adult animals.

To test my hypothesis, I needed to establish an optimal paradigm to induce dependence to CSE in both adolescents and adults. For tests of nicotine dependence, osmotic minipumps are commonly used (Damaj, 2003; Malin et al., 1994; Markou et al., 1998; O'Dell et al., 2004; Shram et al., 2008). These minipumps contain a concentration of nicotine that is diffused into the animals at a known daily concentration. However, I could not use a minipump in my paradigm because the tar in the CSE would clog up the pump. Furthermore, since we are not confident of the stability of the unknown constituents in CSE, it was best to prepare CSE solutions fresh daily. Furthermore, using a daily injection approach would allow us to compensate for adolescent animal growth. In preliminary studies I treated animals with multiple subcutaneous injections daily, according to previously published reports (Balfour et al., 2000; Benwell and Balfour, 1992; Kota et al., 2007). However, animals showed signs of distress due to the excessive number of injections. I therefore switched to intravenous injections, which are not only less stressful, but also a better representation of the route of administration in smoking

than subcutaneous administration, since it takes much less time for drug to reach the brain. The first step was to find an optimal daily dose and volume to induce dependence. After several attempts, 1.5 mg/kg/day given in 3 daily sessions of 15 infusions per session was found to induce a dependence state in adult animals without causing signs of distress.

Having established an appropriate paradigm, I could now test the hypothesis that adult and adolescent animals would show greater spontaneous somatic and affective withdrawal symptoms following chronic treatment with CSE as compared to nicotine. To examine somatic withdrawal, I used a well characterized paradigm of counting physical symptoms of distress displayed in a rat, where the intensity of withdrawal correlates with the frequency to physical symptoms (Malin et al., 1994, 1992). With this exposure regimen, adult rats treated with CSE displayed somatic withdrawal symptoms that emerged as soon as 4 hrs after the last drug exposure, much faster than in those animals exposed to chronic nicotine. Consistent with prior studies (Kota et al., 2007; Natividad et al., 2010; Laura E. O'Dell et al., 2006a; Hugo A. Tejada et al., 2012), nicotine treatment alone did not result in spontaneous somatic withdrawal in adolescent animals. However, adolescents treated with CSE did show significant somatic withdrawal symptoms 18 hrs after the last drug exposure. As hypothesized, the addition of cigarette smoke constituents enhanced nicotine somatic withdrawal in both adult and adolescent rats.

To test affective withdrawal, I used the light-dark box test of anxiety-like behavior, as anxiety is an affective measure of withdrawal (Costall et al., 1989; Carrie E. Wilmouth and Spear, 2006). In this test, anxiety-like behavior is represented as an increase in the time spent on the dark side of the box as compared to saline-treated

controls. I found that both adult and adolescent rats treated with CSE showed anxiety like behavior at 18 hrs after the last drug exposure. However, adult and adolescent rats treated with nicotine did not, confirming that the addition of cigarette smoke constituents enhanced nicotine affective withdrawal in both age groups. Negative mood disturbances such as anxiety can last up to years after smoking cessation, and can often lead to relapse (West et al., 1989). Thus, I wanted to further investigate long-term effects of nicotine and CSE exposure on affective withdrawal. Using the same animals that had undergone the light-dark box test at 18 hrs of withdrawal, I re-tested anxiety-like behavior 30 days after the last drug exposure, this time using center time in an open field as a measure of anxiety. In this test, anxiety is shown by a decrease in the time spent in the center of an open field chamber compared to saline controls. Adolescent animals treated with CSE displayed greater anxiety-like behavior one month after the last drug exposure than adults, whereas animals chronically treated with nicotine alone showed no anxiety at this test interval. These findings show that treatment with CSE induces a persistent anxiety state in adolescent rats. Thus, the withdrawal syndrome that adolescents experience following chronic CSE exposure may be different from that in adults, or in adolescents chronically treated with nicotine alone. This persistent negative affective state may explain the reason why teenagers have a difficult time quitting tobacco use.

Since the nicotine content among the drug groups is equal, these findings show that the non-nicotinic constituents of cigarette smoke are contributing to the neuroadaptations that occur during the formation of dependence, resulting in a more intense withdrawal syndrome upon cessation. To investigate if nAChRs were involved in the enhancement of withdrawal displayed in animals chronically treated with CSE, I used

mecamylamine, a non-selective nAChR antagonist, to precipitate withdrawal. If nAChRs were involved in this enhancement, I would expect an increase in precipitated somatic and affective withdrawal in CSE animals when compared to nicotine animals. The findings showed that both adult and adolescent animals treated with CSE showed higher mecamylamine-precipitated somatic withdrawal than animals treated with nicotine alone. However, mecamylamine did not precipitate affective withdrawal in either age group, regardless of the treatment. The results suggest that the cigarette smoke constituents may enhance somatic withdrawal via a nAChR-based mechanism, whereas the role of nAChRs in enhancing affective withdrawal is less clear.

Together, these findings show the important contribution of non-nicotine cigarette smoke constituents on dependence and withdrawal. Furthermore, my results demonstrate that adolescents are more susceptible to the long-term effects of cigarette smoking, showing negative affective states into adulthood after chronic CSE treatment. Together, this further validates cigarette smoke extract as a better approach to study tobacco dependence in animals than nicotine alone.

Future Directions

Future work may focus on obtaining a deeper understanding of the mechanisms underlying the persistent affective withdrawal observed in adolescent animals treated with CSE. Studies have linked increases in CRF expression within the CeA to increases in affective withdrawal from nicotine (Baldwin et al., 1991; Cohen et al., 2015; George et al., 2007). Thus, future studies could assess regional CRF mRNA expression. In addition,

a blood corticosterone analysis may show increases of the stress hormone in CSE-exposed adolescent animals.

A decrease in DA output into the striatum is also observed during withdrawal (Koob et al., 1993; Natividad et al., 2010). Future studies could also use microdialysis to assess the effect that chronic CSE treatment has on DA neurotransmission in withdrawn animals. Since animals chronically treated with CSE show enhanced withdrawal, I hypothesize that chronic CSE treatment would result in larger decreases of DA transmission in the striatum during withdrawal, independent of age.

Chronic exposure to CSE upregulates nicotinic receptor binding in adult and adolescent rats

As a final stage of my dissertation, I aimed to investigate neuropharmacological adaptations to nAChRs that occur after chronic CSE or nicotine exposure within neurocircuits that mediate the transition from initial tobacco use to dependence and withdrawal, and to do so in adults and adolescents. Clinical data shows that binding to nAChRs is upregulated in heavy smokers (Arthur L Brody et al., 2013b; Marks, 1983; Schwartz and Kellar, 1983). Adult animals with chronic nicotine exposure show similar patterns of upregulation to that of humans (Henderson and Lester, 2015). However, adolescent animals have proven to be resistant to upregulation in binding after chronic nicotine exposure, which may correlate with their observed resistance to nicotine withdrawal symptoms (Counotte et al., 2012; Doura et al., 2008; Laura E. O'Dell et al., 2006a; Trauth et al., 1999). However, I have shown that chronic exposure to CSE manifests in a withdrawal state in adolescent rats, and enhances withdrawal in adult rats. Furthermore, CSE self-administration enhanced drug craving in reinstatement tests (Ch.

2). It has been proposed that increased expression nAChRs in the brain results in craving and relapse during abstinence (Picciotto et al., 2008; Schwartz and Kellar, 1983). Thus, I hypothesized that chronic CSE exposure would result in higher upregulation of nAChR binding in both adult and adolescent rats.

To test this hypothesis I used ^{125}I -epibatidine (\pm cytisine) or ^{125}I - α -bungarotoxin to selectively label $\alpha 4\beta 2$, $\alpha 3\beta 4$, or $\alpha 7$ nAChRs in animals chronically treated with saline, CSE or nicotine. I found that the inclusion of cigarette smoke constituents enhanced upregulation of nAChRs in both adult and adolescent rats. In summary, chronic CSE induced an age-dependent upregulation of $\alpha 4\beta 2$ nAChRs in the MeA and $\alpha 7$ nAChRs in amygdala and LH, and an age-independent upregulation of $\alpha 4\beta 2$ nAChRs in the SN and of $\alpha 3\beta 4$ nAChRs in the MHb and IPN. The amygdala, LH and MHB-IPN pathway are all involved in modulating the rewarding properties of stimuli through regulation of DA output from the VTA and SN and other outputs in the reward system (Watabe-Uchida et al., 2012). Furthermore, within the amygdala, the BLA is involved in craving, a symptom of withdrawal that often leads to relapse (Koob, 2009). Thus, a CSE-induced increase in nAChR binding in the amygdala, LH, SN, MHb and IPN may coincide with the increased craving and withdrawal that was observed in CSE treated animals.

When analyzing age effects, on average, adolescents showed higher binding than adults. However, they did not show limited nAChR upregulation after chronic CSE treatment, as has been reported with chronic nicotine treatment (Doura et al. 2008). In some regions, adolescents even showed greater nAChR upregulation than adults. This suggests that nAChR pharmacology is different between adult and adolescents, and this may influence differences in the networks that mediate drug associated behaviors.

Together, these results provide further evidence that cigarette smoke constituents influence nAChR properties. A better understanding of how these constituents enhance nAChR upregulation will assist in the development of nAChR based pharmacotherapies for smoking cessation that are superior and specifically designed for different age groups.

Future Directions

There are many mechanistic possibilities as to how the non-nicotine constituents in CSE may enhance nicotine-induced upregulation of nAChRs. The term “upregulation” may explain phenomena beyond just receptor number. It may be due to increases in affinity resulting from changes in conformation or stoichiometry of the receptor, reduction in turnover rate or an increase in trafficking of nAChR proteins intracellularly, or a combination of all (Govind et al., 2012, 2009; Henderson and Lester, 2015; Lester et al., 2009; Nelson et al., 2003). Since there are many constituents in CSE, it is possible that they may be acting both directly on the receptor and intracellularly. One possible interaction may be through allosteric modulation, as allosteric modulators have been shown to further upregulate nAChRs (Peng et al, 1994). Allosteric modulation of nAChRs by CSE may be investigated via a competitive radioligand binding assay (Lazareno, 2004). Furthermore, to further investigate whether CSE-induced increases in nAChR binding correlate with increases in receptor subunit expression, an *in situ* hybridization experiment can be used.

General Limitations

There are a few limitations in these experiments. One of the major challenges in studying smoking in animals is using a model that best models smoking in humans. CSE is no exception. The CSE solution precipitated after a couple of days after being made; this did not allow me to use the more commonly used osmotic minipump for nicotine dependence and radioligand binding studies. This does not allow us to fully compare our withdrawal and binding findings with other studies, as the route of administration greatly differs. Nevertheless, I believe passive intravenous administration of CSE is a valid model of smoking as the time it takes for the drug to reach the brain is comparable to that of a smoker.

Another limitation is the composition of CSE. Due to the fact that it is made in saline, an aqueous solvent, CSE contains only the aqueous constituents of cigarette smoke, hence we are not accounting for the other ~ 60% of non-aqueous constituents of cigarette smoke (Schumacher et al., 1977). The extracts commonly used in tobacco research are prepared in an organic solvent in order to dissolve the tar phase of the smoke (Ambrose et al., 2007.; Brennan et al., 2014; Danielson et al., 2014). Because all of my experimental paradigms require intravenous infusion of CSE, an organic solvent was not practical.

Lastly, CSE was made with one brand of cigarettes. Reinforcing effects of smoke extracts have been shown to be brand dependent. One group has shown that reinforcing effects of tobacco particulate matter (TPM), an extract produced from the particulate phase (or “tar” phase) of tobacco smoke, are different between TPM made with commercial cigarettes than with roll-your-own cigarettes (Brennan et al., 2014). Thus, a

future study may include comparing our current findings with those done with other brands of cigarettes.

In spite of these limitations, aqueous CSE is a reliable tool to study tobacco dependence, enough to produce robust behavior on all experimental paradigms used. I have shown that CSE is more potent than nicotine alone in craving induced by cue- and drug-priming. CSE is also more effective than nicotine alone in inducing both somatic and affective withdrawal and upregulation of nAChR binding, in both adult and adolescent rats. Together this shows the increased validity of using CSE, instead of nicotine alone, in preclinical models of tobacco dependence.

General Future Directions

Future studies should investigate gender differences between in the effects of CSE and nicotine on withdrawal and relapse. Studies have shown females are more susceptible to nicotine craving and withdrawal (Kota et al., 2008; Natividad et al., 2013; Torres et al., 2009, 2008). Thus it will be interesting to determine whether cigarette smoke constituents further influence craving and withdrawal in this already susceptible gender group.

Final Conclusions

Taken together I have shown that an animal model of smoking including non-nicotine tobacco constituents is both feasible and more valid than a model based on nicotine alone, as it enhances addictive behaviors in both adult and adolescent rats. My work is the first to show that a history of self-administration of CSE increases nicotine craving and reduces the potency of a potential smoking cessation drug to reduce nicotine- and cue-induced relapse. Furthermore, this work demonstrates that CSE is more potent at

producing somatic and affective withdrawal than pure nicotine in adult and adolescent rats, and that adolescents are more susceptible to the long-lasting effects of CSE-induced affective withdrawal than adults. Lastly, I found that CSE enhances nicotine-induced nAChR upregulation, in some cases more so in adolescents than in adults, adding to the vulnerability that adolescents have on developing tobacco dependence. These findings are important steps towards the development of better medications to treat tobacco dependence that can be designed to treat a specific age group.

Greater Implications

With the recent increase in popularity and consumerism of e-cigarettes, a nicotine delivery device, there is debate about whether they will benefit public health. This prompts me to address the issue that this work may be interpreted to define e-cigarette as “free-of-risk” in adults and adolescents, as the dependence producing potential of nicotine alone is minimal in the studies presented here. However, this work in isolation should not be used to assess the long-term effects of e-cigarette use. E-cigarettes may be a safer alternative than conventional cigarettes as they expose users to fewer toxicants than tobacco (McRobbie et al., 2014), however, the long-term effects of e-cigarette use on health are not known and are not evaluated in this work. Some organizations are hesitant to recommend e-cigarettes for smoking cessation, because of the limited evidence of effectiveness and safety (Products, 2016; Schraufnagel et al., 2014). The National Institute on Drug Abuse raises concern over the possibility that they could perpetuate nicotine addiction and thus interfere with quitting (“DrugFacts: Electronic Cigarettes (e-Cigarettes) | National Institute on Drug Abuse (NIDA),” 2016). This work shows that nicotine alone may indeed prime smokers to crave smoking leading to relapse. There is

an increasing use of e-cigarette among the adolescent population. My work does not look at the effects that adolescent nicotine exposure have on subsequent drug use later in life. Nicotine exposure during periods of significant brain development, such as adolescence, can disrupt the growth of brain circuits that control attention, learning, and susceptibility to addiction (Youth, 2016). Others have found that youth who use e-cigarettes, are more likely to go on to use other tobacco products like cigarettes, or other illicit drugs like cocaine (McQuown et al., 2007; Rigotti et al., 2015; Yuan et al., 2015). Thus, although e-cigarettes may be a safer alternative to conventional cigarettes; the findings presented in this work should not be used to make the assumption that they are without negative consequence, especially in the adolescent population, and thus strict regulation to control the use of e-cigarette use among adolescents must remain in effect.

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