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Establishing a Proper Model of Tobacco Dependence: Influence of Age and Tobacco Smoke Constituents

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in Pharmacology

By

Candice A. Gellner

Dissertation Committee:
Professor Frances M. Leslie, Chair
Adjunct Professor James D. Belluzzi
Assistant Professor Christie D. Fowler

2017

DEDICATION

*To **my mother**, my best friend and role model, who gently nudged me to strive for bigger and better things that I never knew were possible. To **my father**, who doesn't really know what a PhD is, but supports me in any way possible.*

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Acknowledgements

There have been many people who have been influential throughout my journey in graduate school and helped to make this dissertation possible. I am forever grateful for their guidance, help, and support.

I would like to thank my advisor, Dr. Frances M. Leslie. If she hadn't challenge me to do less and think more, I would not be writing this document. She not only challenged me intellectually but her guidance and support as my advisor helped develop my skills as a scientist. Thanks to her, I can now say that I am a critical thinker.

Dr. Jim Belluzzi has been instrumental in helping with my statistical analysis and manuscript revisions. I will always have fond memories of our conversations about statistics. I also truly enjoyed getting to know Dr. B on a personal level and will forever cherish our conversations about tennis, choir, and his grandson, who will be a famous tennis player one day! ☺

I would also like to thank my committee members Dr. Cathy Cahill and Dr. Christie Fowler for their invaluable support and guidance throughout my graduate career.

Dr. Phong Luong has been extremely influential during my time at UCI. Phong's guidance helped me pass my advancement, publish my paper, and ultimately get my PhD. Without him I do not believe my time at UCI would have been as successful or bearable. I am extremely thankful that my professor, Dr. Leslie, suggested that I see him. Our conversations have been extremely helpful and without him, I wouldn't be getting my PhD.

I would like to thank both former and present Leslie Lab members which whom I had the pleasure of working with and getting to know personally. In particular I would like to thank Armando for teaching me the ins and outs of self-administration, Menglu for her thoughtful words of advice and science communication focus, Daisy for being a fantastic experiment buddy, and Sarah for being a great friend. I am very lucky that I had the opportunity to work with some really awesome people that have enriched my graduate studies. Without their support, I again would not be writing this document.

I would like to thank all of the undergraduates that have worked with me in both the past and present. They have been instrumental in helping me complete all of the experiments that are part of my dissertation. Michelle, our lab technician, was a crucial partner in helping me collect data for Chapter 4.

I would like to also thank my family and friends for their love and support throughout this process. I am forever grateful for my awesome parents who have helped me through some difficult times in my graduate studies. Their love and encouragement helped keep me going. My wonderful friends (Brandon, Sarah, Kelly, Ali, Kristen, Elyse, Nayna, to name a few) have helped me keep my sanity on numerous occasions. To my boyfriend, who has enriched my life in so many ways and helped me realize that maybe I am actually a nerd. THANK YOU. Without each and every person that has influenced my life the last five years, I would not be getting my PhD today!

Finally I would like to thank UCI for providing travel awards (DECADE, SOM, and Dept. of Pharmacology) so that I could attend conferences such as SfN and EB.

Curriculum Vitae

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EDUCATION

University of California, Irvine, Irvine, CA, 92617
       PhD in Pharmacological Sciences   May 2017
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       B.S. in Biochemistry   May 2012

TEACHING EXPERIENCE

General Chemistry Laboratory Teaching Assistant: University of California, Irvine, 2013
- Instructed students on how to perform experiments and aided the students in problem solving during the lab. Also held office hours to answer questions that students may have about the labs performed or upcoming labs.

Workshop Presenter: Expanding Your Horizons, CSU Sacramento, 2009-2011
- Informational talks about experiments to be performed as demos and hands-on experiences for middle school students. Experiments focused on the chemistry behind ideas as abstract as magic to more practical entities such as the microwave.

RESEARCH EXPERIENCE

Graduate Research:
    Department of Pharmacology, UC Irvine, Spring 2013 – May 2017 (research adviser: Dr. Frances Leslie)
- Understanding how age and tobacco smoke constituents affect animal models of tobacco dependence

    Department of Pharmaceutical Sciences, UC Irvine, Winter 2012 (research adviser: Dr. Young Jik Kwon)
- Investigating liposomes as targeted drug delivery systems

    Department of Molecular Biology and Biochemistry, UC Irvine, Fall 2012 (research adviser: Dr. Celia Goulding)
- Warfarin inhibition of M. tuberculosis DsbG when complexed with M. tuberculosis VKOR

Undergraduate Research:
    Department of Internal Medicine, Hematology and Oncology, UC Davis, 2010-2012 (research adviser: Dr. Paul Henderson)
- The use of nanolipoprotein (NLP) particles to study over expressed proteins in breast cancer.

Department of Chemistry, CSU Sacramento, 2010-2012 (research adviser: Dr. Linda Roberts)
- Investigation, isolation, and characterization of cross-linking L178H ApoA1.

Center for Biophotonics Science and Technology, UC Davis, 2009-2010 (research adviser: Dr. Paul Henderson)
- Investigation of microdosing as a form of cancer treatment.
- Investigation of the use of micelles as a drug delivery platform.
- Using NLPs to study breast cancer.

Lawrence Livermore National Laboratory (LLNL), Summer 2011 (research adviser: Dr. Matthew Coleman)
- Investigated truncations of apolipoproteins and their use in NLP particles.

PUBLICATIONS

Published:
- He, W., Scharadin, T.M., Saldana, M., **Gellner, C.**, et al. (**2015**) Cell-free expression of functional receptor tyrosine kinases. *Sci. Rep. 5*. doi:10.1038/srep12896
- **Gellner, C.A.,** Belluzzi, J.D., and Leslie F.M. (**2016**) Self-administration of nicotine and cigarette smoke extract in adolescent and adult rats. *Neuropharmacology*, 109, 247-253. doi:10.1016/j.neuropharm.2016.06.026
- **Gellner, C**., Reynaga, D. and Leslie F.M. Cigarette Smoke Extract: A preclinical model of tobacco dependence. *Curr. Protoc. Neurosci.* 77:9.54.1-9.54.10. doi: 10.1002/cpns.14

PRESENTATIONS

- *"Adolescent rats display stress- and stress+cue-induced reinstatement after cigarette smoke extract or nicotine self-administration"*
  Poster, SfN, San Diego Convention Center, San Diego, CA   2016

- *"Comparing cigarette smoke extract and nicotine seeking behavior after exposure to stress in adolescent rats"*
  Poster, EB, San Diego Convention Center, San Diego, CA   2016
- *"Age and dose are important factors in the acquisition phase of cigarette smoke extract self-administration"*
  Poster, SfN, McCormick Place Convention Center, Chicago, IL   2015

- *"Self-administration of cigarette smoke extract vs. nicotine alone in adolescent and adult male rats"*
  Poster, SfN, Walter E. Washington Convention Center, Washington, DC   2014

- *"Concentration dependence of cross-linking L178H apolipoprotein A-I"*
  Poster, ACS Undergraduate Symposium, Mills College, Oakland, CA   2012

- *"Obtaining access to membrane proteins using cell-free technologies and nanolipoproteins"*
  Poster, SACNAS National Conference, Convention Center, San Jose, CA   2011

- *"Obtaining access to membrane proteins using cell-free technologies and nanolipoproteins"*
  Scientific abstract and presentation, CBST, Davis, CA   2011

- *"Ex Vivo Synthesis of ErbB2/Her2 NLPs for Biochemical, Structural and Drug Development Studies"*
  Scientific abstract and presentation, CBST, Sacramento, CA   2010

AWARDS & HONORS

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    SACNAS National Conference   October 2011
2011 Partnerships for Innovation Commercialization Plan Award
    Accelerated Medical Diagnostics (AMD)   May 2011
Best Presentation Award
    Center for Biophotonics Science and Technology (CBST)   June 2010 – Sept. 2010

CERTIFICATIONS

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- Public Speaking for Graduate Students, UC Irvine   2013

PROFESSIONAL LEADERSHIP ACTIVITIES & SERVICE

- *Senior Technology Transfer Fellow:* Invention Transfer Group, UC Irvine   2016 – present
- *Managing Editor:* The Loh Down on Science, NPR/KPCC   2016 – present
- *Student Representative:* Department of Pharmacology, UC Irvine   2015 – present
- *Trainee Council and Member:* GPS BIOMED, UC Irvine   2014 – present
- *Education Chair and Member:* DECADE Student Council, UC Irvine   2013 – 2016
- *Laboratory Assistant:* Assist special needs students in their chemistry lab, CSU Sac.   2011
- *President and Member*: CSU Sacramento Chemistry Club   2008 – 2012

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American Association for the Advancement of Science – AAAS
Graduate Professional Success in the Biomedical Sciences - GPS-BIOMED
Association for University Technology Managers - AUTM

ABSTRACT OF THE DISSERTATION

Establishing a Proper Model of Tobacco Dependence: Influence of Age and

Tobacco Smoke Constituents

By

Candice Ann Gellner

Doctor of Philosophy in Pharmacology

University of California, Irvine, 2017

Professor Frances M. Leslie, Chair

Cigarette smoking is the leading preventable cause of death in the United States. Of those who smoke, 9 out of 10 report trying their first cigarette before the age of 18. Although most people who initiate tobacco use are teenagers, animal models for studying tobacco dependence have traditionally focused on how adult animals initiate, withdrawal from and relapse to cigarette smoking. Furthermore, cigarette smoke contains more than 7,000 constituents, including nicotine, yet pre-clinical research has focused on nicotine alone. Our lab began studying these constituents by creating cigarette smoke extract, CSE, a solution which contains the aqueous constituents present in cigarette smoke. Previous work from our lab found that CSE is more potent than nicotine alone and can enhance stress-induced reinstatement in adult male rats. In order to understand how the presence of tobacco smoke constituents may affect adolescents, I investigated the role of these constituents in models of smoking initiation and relapse. I found that the tobacco smoke constituents did not influence adolescent or

adult acquisition of self-administration. Adolescents self-administered more low-dose nicotine than adults when their increased non-specific responding was corrected for. During reinstatement of drug seeking, I found that CSE enhanced stress- + cue-induced reinstatement of drug-seeking behavior with no effect of age. To investigate the role of tobacco smoke constituents on attenuation of adolescent self-administration, a novel smoking cessation pharmacotherapy, AT-1001, which is a selective α3β4 nAChR functional antagonist, was used. AT-1001 attenuated both CSE and nicotine self-administration in adolescent rats to a similar degree. My results suggest that both age and the presence of tobacco smoke constituents are important factors in establishing a proper model of tobacco dependence. Furthermore, my findings provide novel insights on adolescent initiation and relapse and offer an exciting potential for the development of a new tobacco dependence animal model that could help create innovative therapeutics to curb the addiction faced by many.

Chapter 1: Introduction

Health impact of smoking

Cigarette smoking is the single largest cause of preventable disease and death in the United States (Alberg et al 2014). Among the many problems that smoking can cause, it has been shown to increase the risk for coronary heart disease, stroke, and lung cancer (Center for Disease Control and Prevention (CDC) 2014)(Alberg et al 2014). The development of these diseases typically leads to death, which is reflected in the staggering 6 million people a year worldwide that die from tobacco use (World Health Organization 2015). In the United States, cigarette smoking is responsible for one in five deaths annually, or 1,300 deaths every day (Center for Disease Control and Prevention (CDC) 2014). Despite these well-known facts, every day roughly 3,200 people under the age of 18 try their first cigarette (Alberg et al 2014).

Although conventional cigarette use by teenagers has declined, the rates of electronic cigarette (e-cigarette) use increased by 900% over a span of 4 years (3% in 2011 to 27% in 2015) (Surgeon General 2016; Singh et al 2016). E-cigarettes are electronic nicotine delivery systems (ENDS) intended to deliver nicotine to the brain without the toxic byproducts of combustion while retaining the same rewarding effects as cigarettes that make them so profitable, pleasurable, and addictive (Cobb et al 2010). E-cigarettes are marketed as safer alternatives and smoking cessation aids, which lacked federal regulation until 2016 (Paradise 2014). The original lack of regulation led to rapid increases in

youth e-cigarette initiation, with as many as one-third having never smoked a conventional cigarette (Grana et al 2014). Both non-smokers and current smokers use e-cigarettes, with high levels of dual use observed in youth (Grana et al 2014). Furthermore, e-cigarettes are thought to increase the likelihood of continuing and further increasing youth tobacco use (Dutra and Glantz 2014). The recent regulation limits youth (under 18) e-cigarette use with the hopes of minimizing nicotine exposure in teenagers (Kalkhoran and Glantz 2016). However, only future studies will tell if youth e-cigarette use actually decreases.

Teen smoking causes immediate and long-term damage, physically as well as mentally (Centers for Disease Control and Prevention (CDC) 2012). The damage caused by smoking has the potential to prematurely kill 5.6 million of today's youth in America (Alberg et al 2014). Of the 42.1 million people who smoke, 9 out of 10 smokers report trying their first cigarette by the age of 18 (Center for Disease Control and Prevention (CDC) 2014) On average, smokers who start in their teens use more tobacco, are more likely to develop nicotine dependence, have a harder time quitting, and are more likely to relapse than those who begin as adults (Breslau and Peterson 1996; Kandel and Chen 2000; Cui et al 2006). Therefore, understanding the unique effects of smoking during adolescence is critical for treating the adverse health consequences and preventing premature death.

Adolescence

Adolescence is a transition period between childhood and adulthood, with conservative reports estimating ranges from 12-18 years in humans and postnatal day (P) 28-42 in rodents (Spear 2000). More recent literature suggests that changes which signal adolescent onset occur as early as 10 in humans or P21 in rats with maturation lasting till the mid 20s in humans or around P55 in rodents (Laviola et al 2003; Sturman and Moghaddam 2011; Burke and Miczek 2014; Yuan et al 2015). Adolescence is a developmental period that is highly conserved across mammalian species, and both humans and rodents experience similar behavioral and physiological changes (Spear 2007; Spear 2013; Yuan et al 2015). During this critical time, adolescents exhibit increased risk-taking, novelty-seeking, and peer association (Spear 2013). Adolescence is also a period of vulnerability for the onset of substance abuse (Lubman et al 2007).

Structural and neurochemical maturation of the brain during adolescence underlies the behavioral characteristics of this developmental period. These changes during adolescence parallel increases in functional neuronal connectivity (Luna et al 2010; Yuan et al 2015), when organization of local interactions shifts to a more distributed connectivity by young adulthood (Fair et al 2009; Hwang et al 2010; Satterthwaite et al 2013; Yuan et al 2015). Connections that undergo adolescent maturation include the prefrontal cortex (PFC) to nucleus accumbens (NAc) and the amygdala to PFC pathways (Cunningham et al 2002; Brenhouse et al 2008). These pathways play important roles in cognition, mood, reward and motivated behavior (Ernst and Fudge 2009).

The triadic circuit between the PFC, NAc, and basolateral amygdala (BLA) regulates executive control of reward and motivated behavior (Ernst and Fudge 2009), which is still nascent during adolescence. In early adolescence, projections from the BLA to the PFC are immature and the NAc develops earlier than the associated PFC regions, which consequently leads to increased novelty-seeking and risk-taking behaviors (Cunningham et al 2002; Galvan et al 2006; Ernst and Fudge 2009). These maturations and remodeling events label adolescence as a critical period of vulnerability for the initiation of drug abuse and addiction (Chambers et al 2003; Adriani and Laviola 2004; Anker and Carroll 2010).

In addition to structural changes, the adolescent brain undergoes neurochemical changes with distinct maturation and substantial reorganization of the dopamine (DA) system that lasts until early adulthood (Wahlstrom et al 2010; O'Donnell 2010; Yuan et al 2015). The DA system plays a crucial role in the development of associative learning and motivated behavior (Cunningham et al 2002; Chambers et al 2003). In adolescents, the levels of DA are lower in the ventrolateral striatum, but are the highest in the PFC and NAc (Goldman-Rakic and Brown 1982; Cao et al 2007; Philpot et al 2009). Before the end of adolescence, DA receptor levels peak in the striatum and PFC, and then stabilize at lower adult levels (Teicher et al 1995; Tarazi and Baldessarini 2000; Andersen et al 2000).

Along with neurochemical changes that take place during adolescence, come changes to neuronal nicotinic acetylcholine receptors (nAChRs). Naïve

adolescent animals have been shown to have greater binding of β2 and α7 nAChR subtypes compared to adults (Doura et al 2008). Unpublished data from our lab has shown that adolescents also have higher α3β4 nAChR binding in the interpeduncular nucleus (IPN). These studies indicate that the adolescent brain is distinctly different from the adult brain and make adolescence a particularly vulnerable period to drug-induced alterations.

Nicotine and non-nicotine constituents of tobacco smoke

More people in the United States are addicted to tobacco than to any other drug (American Society of Addiction Medicine 2008). With the advent of e-cigarettes, more people, especially teenagers, are being exposed to nicotine. Nicotine is the primary psychoactive component in cigarettes and has been traditionally implicated as the component responsible for tobacco dependence (Benowitz 1988; Stolerman and Jarvis 1995; Harvey et al 2004). Nicotine acts on nAChRs to release DA and other neurotransmitters producing a euphoric/positive feeling. After a period of about 2 hours, the euphoric feeling dissipates as nicotine is metabolized in the liver by CYP2A6 and CYP2B6 (Benowitz et al 2009). The development of addiction is thought to consist of positive (euphoria) and negative (withdrawal) reinforcement, with the positive reinforcement driving the beginning stages and a switch to negative reinforcement driving the later stages of the addiction cycle (Koob and Volkow, 2010).

Although nicotine is the primary psychoactive constituent in cigarettes, a growing clinical literature implicates the other components of tobacco smoke as

additional contributors to addiction (Alpert et al 2016). Smoking has been shown to produce inhibition of both types (A and B) of monoamine oxidase (MAO), with smokers having 20-40% lower MAO activity than non-smokers (Berlin and M. Anthenelli 2001). Inhibiting MAOs that break down monoamine neurotransmitters such as DA may lead to a potentiation of nicotine's effects (Berlin and M. Anthenelli 2001). Clinical evidence also suggests that the non-nicotine constituents of cigarette smoke provide reinforcing sensory stimulation and minimize excessive irritation (Rose 2006). A clinical study, in which smokers subjectively compared denicotinized cigarettes to intravenous nicotine infusions, reported that denicotinized cigarettes reduce their cravings and were significantly more satisfying than the no smoking conditions (Rose et al 2000). These clinical studies provide further support for the idea that the non-nicotine constituents act as reinforcers and illustrate the importance of studying the non-nicotine constituents in tobacco addiction.

Animal Models of Tobacco Dependence – Reward/Acquisition

Laboratory animals provide a way to model and test the several stages of tobacco dependence, which are experienced by humans. The modeling of tobacco dependence in animals involves three distinct stages: drug use initiation or acquisition, withdrawal, and relapse or reinstatement of drug seeking (Lynch et al 2010). Tobacco dependence begins with drug use initiation, or first exposure to a drug. As in humans, drugs such as nicotine have been shown to elicit positive reinforcing effects in animals (Corrigall and Coen 1989).

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Although nicotine is self-administered by animals this may not accurately represent the addictive nature of smoking. Compared to other drugs of abuse, nicotine is only weakly reinforcing and requires very specific parameters for acquisition of self-administration (Donny et al 1998). When given the choice, animals always choose cocaine over nicotine (Manzardo et al 2002) and will not self-administer nicotine when substituted for cocaine (Mello and Newman 2011). Furthermore, the common dose used for nicotine self-administration is 30 µg/kg/infusion (free base), which is equivalent to the amount of nicotine a smoker receives when smoking a single cigarette (Miller et al 1977; Rose and Corrigall 1997; Benowitz 2007). Even though metabolism in rats is twice as fast as humans ($t_{1/2}$=45 in rats and 2 hrs in humans, (Matta et al 2007)), repeated i.v. doses at 30 µg/kg/infusion would still produce higher blood levels of nicotine than a smoker would generally receive in smoking a single cigarette.

Nicotine self-administration has traditionally been studied in adult animals, yet the majority of people start smoking as teenagers. More recent studies have begun to explore age-specific effects of nicotine. An acute injection of nicotine enhances locomotion in adolescents, but decreases locomotion in adults (Cao et al 2010). Adolescent rats readily acquire nicotine self-administration and take more nicotine than adults (Faraday et al 2003; Levin et al 2003; Chen et al 2007; Levin et al 2007; Levin et al 2011; Natividad et al 2013). In conditioned place preference (CPP), early adolescent rats display enhanced sensitivity to the rewarding effects of nicotine (Vastola et al 2002; Belluzzi et al 2004; Shram et al 2006; Kota et al 2007; Brielmaier et al 2008; Torres et al 2008). Adolescents

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given high doses of nicotine do not find it aversive, as compared to adults (Fudala et al 1985; Brielmaier et al 2008), and adolescent exposure to nicotine lowers aversion to high doses of nicotine in adulthood (Torres et al 2008). Furthermore, adolescent nicotine exposure results in long lasting behavioral and neurochemical changes that enhance reward sensitivity to other drugs of abuse (McQuown et al 2007; Dao et al 2011; Mojica et al 2014). Taken together, these studies suggest that adolescent nicotine exposure produces unique behavioral responses, which may alter brain maturation and development.

Mechanisms Underlying Reward/Acquisition

Nicotine's rewarding effects are dependent on the mesolimbic DA system (Koob 1992; Rose and Corrigall 1997) and produce behavioral responses such as nicotine self-administration. Nicotine self-administration is mediated by regulation of DA release in the ventral striatum (Picciotto et al 1998; Gotti et al 2010). Nicotine dose-dependently increases DA release in the ventral and dorsal striatum, with significant age differences in efficacy and potency that are specific to the ventral striatum (Azam et al 2007). Chronic nicotine treatment in adolescent rats increases DA transporter densities in the caudate putamen and NAc (Collins et al 2004). Furthermore, nicotine enhances DA release in the NAc shell to a greater extent in adolescents as compared to adults (Shearman et al 2008). Adolescent nicotine exposure also induces changes in the serotonin system. Acute nicotine increases extracellular serotonin overflow in the NAc shell, while decreasing DA and serotonin in adolescent mPFC (Shearman et al

2008; Yuan et al 2015). Nicotine has also been shown to alter limbic regions via a serotonin 1A receptor mechanism (Dao et al 2011). These preclinical studies suggest important age-dependent effects of nicotine on mesolimbic DA and serotonin systems.

Nicotine-induced changes also take place at the receptor level and have been shown to alter behavioral responses. Chronic nicotine exposure has been shown to have differential effects on adolescent and adult nAChR binding. Adults, but not adolescents, show enhanced binding at β2* and α7 nAChRs after chronic nicotine exposure (Doura et al 2008). Furthermore, chronic nicotine exposure selectively up-regulates α4β2-like binding with minimal effect on α3β4 binding in adults (Nguyen et al 2003).

To further the understanding of these mechanisms, studies have used pharmacological agents to modulate nAChRs and block nicotine-induced reward/acquisition. Antagonists at the nAChR such as mecamylamine (a non-selective nAChR antagonist) and dihydro-beta-erythroidine (DHBE, an α4β2 nAChR antagonist) have been shown to attenuate nicotine self-administration in rats (Watkins et al 1999). Varenicline (Chantix), a commercially available smoking cessation agent, has been shown to partially activate α4β2 receptors with the overall outcome of attenuating nicotine self-administration (O'Connor et al 2010; Le Foll et al 2012; Costello et al 2014). In addition to blocking nicotine self-administration, varenicline dose-dependently enhances responding for nonpharmacological reinforcers (Levin et al 2012) and is self-administered on its own (Cippitelli et al 2015). The ability of varenicline to produce nicotine-like

effects may be why Chantix is only successful in helping 23% of smokers stay abstinent for over a year. In order to create a more efficacious smoking cessation agent, a new class of drugs has been created, with AT-1001 as its prototype. AT-1001 targets α3β4 nAChRs as a functional antagonist and has been shown to dose-dependently attenuate nicotine self-administration (Toll et al 2012) without being self-administered on its own (Cippitelli et al 2015). As with all other nicotine self-administration studies, these drugs have only been shown to attenuate nicotine self-administration in adult rats. In order to curb the addiction which beings in adolescence, it is important to understand how these drugs may attenuate adolescent nicotine self-administration.

Non-Nicotine Constituents of Tobacco Smoke in Reward/Acquisition

Although nicotine has been shown to produce distinct behavioral effects, it does not accurately represent the addictive nature of tobacco use. The use of nicotine alone in self-administration studies lacks the more than 7,000 other constituents that are present in cigarette smoke (NTP, 2014). To address this problem, studies have begun to investigate the role of non-nicotine constituents, either alone or in combination with nicotine. Tobacco alkaloids, a group of compounds that are structurally similar to nicotine, have been shown to increase locomotor activity, have reinforcing effects alone and differential effects on nicotine self-administration (Clemens et al 2009; Caine et al 2014; Hall et al 2014; Costello et al 2014).

Other constituents found in tobacco smoke are monoamine oxidase inhibitors (MAOIs), which inhibit the breakdown of monoamine neurotransmitters such as DA and add to the reinforcing effects of tobacco (Fowler et al 1996; Lewis et al 2007). To model MAO inhibition, our lab and others have used an irreversible and non-selective MAOI, tranylcypromine (TCP), which is not present in tobacco smoke. Pretreatment with TCP promotes low dose nicotine self-administration (Villégier et al 2007; Smith et al 2015) and acute treatment with TCP results in an increase in DA transmission, which has been shown to serve a critical role in nicotine reinforcement (Villégier et al 2006; Villégier et al 2007; Villégier et al 2011). Another irreversible but selective MAOI, clorgyline, has been shown to enhance nicotine self-administration (Guillem et al 2005; Guillem et al 2006). In addition, pretreatment with norharmane, a beta-carboline MAOI, which is naturally found in tobacco smoke, increases nicotine self-administration (Poindexter and Carpenter 1962; Guillem et al 2005). Our lab has also studied norharmane and found that rats acquire self-administration of norharmane alone and the reinforcing effects of norharmane and nicotine are additive (Arnold et al 2014). These studies demonstrate that the non-nicotine constituents have distinct reinforcing properties that may better reflect the addictive nature of smoking.

As with nicotine self-administration, most studies investigating the non-nicotine constituents in tobacco smoke have focused on adult behavior. To my knowledge, only a few studies have investigated the role of tobacco smoke constituents during adolescence, yet this is the period in which most humans initiate drug use. Our lab has used TCP to model MAO inhibition and has found

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that the TCP's enhancement of nicotine self-administration was not age-dependent (Villégier et al 2007). Our lab also investigated acetaldehyde, a major constituent of tobacco smoke, and found that it enhanced the acquisition of adolescent but not adult nicotine self-administration (Belluzzi et al 2005). The effects of passive cigarette smoke exposure during adolescence have also been investigated. In CPP models, pre-exposure to cigarette smoke enhanced the rewarding effects of nicotine in adulthood as well as at high doses during adolescence (la Peña et al 2014; la Peña et al 2015). However, pre-exposure to cigarette smoke or nicotine was shown to reduce both adolescent and adult nicotine self-administration (la Peña et al 2014). No studies to date have examined aqueous smoke extracts during this important developmental period. To most accurately model initiation of drug use, studies should focus on the role of non-nicotine tobacco smoke constituents during adolescence.

Animal Models of Tobacco Dependence – Craving/Relapse

Tobacco addiction is a chronic relapsing disorder in which the craving to smoke outweighs the negative consequences and the desire to quit (Koob and Volkow 2010; Lynch et al 2010; Bauzo and Bruijnzeel 2012). The factors that cause humans to relapse include the exposure to stimuli that the smoker associates with the positive rewarding effects, the negative emotional states of withdrawal which include stress, and changes in their urge to smoke, with the most important factor for relapse being a persistent and increased urge to smoke (Doherty et al 1995; Swan et al 1996; Shiffman et al 2002; Koob and Volkow

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2010; Zaveri et al 2015). To model this human phenomenon, animals are tested using a self-administration extinction/reinstatement paradigm.

Extinction/reinstatement of self-administration is used to model craving and human relapse to smoking. The reinstatement model has face validity as the triggers that cause humans to relapse can reliably reinstate drug-seeking behavior in laboratory animals (Shaham et al 2003). The paradigm begins with drug exposure, where animals are allowed to stably respond before removal of the drug, called extinction. Once responding for the drug ceases, exposure to certain triggers causes reinstatement of drug seeking. These triggers include stress via footshock or a pharmacological stressor, yohimbine, visual and/or auditory cues associated with intake, or a priming injection of the self-administered drug. To assess reinstatement of drug seeking, responding on the drug-associated lever is measured, although no drug is given when the lever is activated.

Animal models of nicotine reinstatement do not fully represent the intensity of triggers that cause relapse to smoking. Reinstatement using a priming injection of nicotine alone has produced mixed results, where one study has shown that nicotine can reinstate nicotine-seeking behavior (O'Connor et al 2010) whereas others demonstrate the need to add previously paired drug cues (LeSage et al 2004; Shram et al 2008a; Feltenstein et al 2012).

Other triggers that more reliably reinstate nicotine-seeking behavior are stress and the presentation of cues that were previously paired with nicotine.

Stress, whether footshock or yohimbine, has been shown to readily reinstate nicotine-seeking behavior in adults (Buczek et al 1999; Bruijnzeel et al 2009; Feltenstein et al 2012). The presentation of cues that were previously paired with nicotine can also reliably reinstate nicotine-seeking behavior (LeSage et al 2004; Bespalov et al 2005; O'Connor et al 2010; Feltenstein et al 2012). When triggers such as drug priming or stress are combined with cues, mixed results are seen where some groups report an enhancement of drug-seeking behavior (Schenk et al 2008; Feltenstein et al 2012), while others show that cues do not enhance nicotine-primed reinstatement (LeSage et al 2004).

Reinstatement of nicotine-seeking behavior in adolescent rats is extremely understudied. In one study, a priming injection of nicotine compared to saline was shown to reinstate nicotine-seeking behavior independent of age or strain (Shram et al 2008a). To my knowledge, there are no studies that look at the effects of stress- or cue-induced reinstatement of nicotine-seeking behavior in adolescents. Studies investigating the reinstatement to cocaine-seeking show that adolescents are more sensitive to high doses of cocaine-induced reinstatement, do not reinstate to cues alone, and show enhanced reinstatement after an injection of yohimbine compared to adults (Anker and Carroll 2010). Although this cocaine study points to important age differences in reinstatement to cocaine-seeking behavior, the factors that affect reinstatement to nicotine-seeking behavior have yet to be fully studied.

Mechanisms Underlying Craving/Relapse

Mechanism-based studies attempt to understand the involvement of nAChRs in reinstatement of nicotine-seeking behavior in adult rats. An α4β2 nAChR partial agonist, varenicline, has been shown to block nicotine-, cue-, or the combination of nicotine- and cue-induced reinstatement of nicotine-seeking behavior (O'Connor et al 2010; Le Foll et al 2012), while others have shown that varenicline does not block cue-induced reinstatement (Wouda et al 2011). These discrepancies in results are dependent of the methods used to induce reinstatement. Mecamylamine, a non-selective and non-competitive antagonist of nAChRs, has been shown to attenuate cue-induced reinstatement of nicotine seeking (Liu et al 2007). Other studies of cue-induced reinstatement have pointed to the involvement of α7 nAChRs, either alone or in complex with NMDA receptors (Li et al 2012; Liu 2014). AT-1001, a selective, high-affinity α3β4 nAChR functional antagonist, has been shown to attenuate nicotine- and stress- + cue-induced reinstatement of nicotine seeking (Zaveri et al 2015). These studies suggest that nicotine exposure in adults causes long-lasting changes to nAChRs, since partial agonists and antagonists of these receptors can block reinstatement of drug seeking, even when nicotine is no longer present. To date, no studies have investigated how these nAChR ligands may influence smoking initiation or relapse during adolescence.

Non-Nicotine Constituents of Tobacco Smoke in Craving/Relapse

Our lab has begun to investigate the role of non-nicotine constituents in animal models of relapse using aqueous cigarette smoke extract (CSE). Adult rats that were primed to reinstate with CSE do not rely on cues to reinstate CSE-seeking behavior whereas animals that were primed to reinstate with nicotine reinstated nicotine-seeking behavior only when cues were present (Reynaga 2015). Similarly, adult animals that had self-administered CSE robustly reinstated drug-seeking in response to yohimbine-induced stress without the presentation of cues, whereas animals that previously self-administered nicotine alone required cues to reinstate nicotine-seeking behavior (Costello et al 2014). These results indicate that the non-nicotine tobacco smoke constituents in CSE may contribute to smoking relapse.

Rationale for Current Studies

Preclinical studies have traditionally focused on adult male rats to model tobacco dependence, while the clinical literature suggests that adolescence is an important developmental period in which initiation of tobacco use occurs. In addition, animal studies have concentrated on nicotine alone although there are more than 7,000 constituents in cigarette smoke (National Toxicology Program (NTP) 2014). To close the gap between the preclinical and clinical literature, our lab has created a CSE self-administration model to study the aqueous non-nicotine constituents and their potential role in tobacco use. To date, the lab has shown that CSE is a more potent reinforcer than nicotine alone and that it

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enhances reinstatement of drug seeking in adult male rats, suggesting that it may be a better model for tobacco dependence. My specific aims set out to extend the study of CSE to adolescent animals and to delineate the role of the non-nicotine tobacco smoke constituents during initiation of drug use and relapse. These findings will provide novel insights on adolescent initiation and relapse and offer an exciting potential for the development of a new tobacco dependence animal model that could help create innovative therapeutics to curb the addiction faced by many. It will also provide insights into whether or not e-cigarettes and tobacco differ in their impacts on teens.

Chapter 2: Tobacco Smoke Constituents Do Not Alter Self-Administration in Adolescent Male Rats

Introduction

Tobacco use is the leading preventable cause of death worldwide, killing more than 6 million people a year (World Health Organization 2015). Smoking is an adolescent-onset disorder, with almost 90% of smokers trying their first cigarette by the age of 18 (Center for Disease Control and Prevention (CDC) 2014). Although current rates of conventional cigarette use have markedly declined, the use of electronic nicotine delivery systems (e-cigarettes) among school-age children has tripled in the last year (Singh et al 2016). E-cigarettes, which are marketed as safer alternatives and smoking cessation aids, may actually increase the likelihood of continuing and increasing tobacco use among adolescents (Dutra and Glantz 2014).

Adolescence is characterized as a period of development when individuals demonstrate risk-taking and novelty seeking behaviors (Spear 2000). Both clinical (Chen and Millar 1998; Everett et al 1999) and preclinical (Vastola et al 2002; Belluzzi et al 2004; Brielmaier et al 2008) studies have found adolescents to be more sensitive to the rewarding properties of nicotine. Adolescent rats have been shown to acquire nicotine self-administration more readily, and to take more nicotine, than adults (Levin et al 2003; Chen et al 2007; Levin et al 2007). In conditioned place preference, rats in early adolescence display enhanced sensitivity to the rewarding effects (Vastola et al 2002; Belluzzi et al 2004;

Brielmaier et al 2008), and reduced sensitivity to the aversive effects of nicotine (Wilmouth and Spear 2004; Shram et al 2006; Torres et al 2008).

Cigarette smoke contains more than 7,000 constituents; hundreds of which are harmful, and about 60 are known to cause cancer (National Toxicology Program (NTP) 2014). However, animal models of tobacco dependence have traditionally examined only the effects of nicotine (Donny et al 1995), the main psychoactive component of tobacco (Stolerman and Jarvis 1995). Some studies have begun to look at the non-nicotine constituents found in cigarette smoke to understand how they may affect nicotine self-administration. Biologically active components such as monoamine oxidase inhibitors have been shown to increase nicotine self-administration (Guillem et al 2005; Villégier et al 2006; Villégier et al 2007; Arnold et al 2014). Acetaldehyde, a combustion product of tobacco, also enhances nicotine self-administration in adolescent, but not adult, rats (Belluzzi et al 2005). Although these findings show that single constituents interact with nicotine, they exclude most tobacco smoke constituents and ignore the possible interactions that may occur between them. In order to study these interactions, we have created a model in which the behavioral effects of aqueous cigarette smoke extract (CSE) are examined. Previous work by our group has shown that CSE is more potent than nicotine alone in adult male rats during the acquisition and maintenance phases of self-administration, and yields sensitized reinstatement to stressors (Costello et al 2014). Based on these studies, I hypothesized that CSE would enhance both adolescent and adult acquisition of self-administration compared to nicotine alone.

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To further our understanding of the mechanisms underlying tobacco use, we have now compared the acquisition of self-administration of nicotine or CSE at varying doses in adolescent and adult male rats. As initiation of smoking typically occurs during adolescence, it is important to study this period of development in animal models of tobacco dependence.

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 by bubbling smoke from commercial cigarettes (Camel unfiltered, RJ Reynolds) through sterile saline, using a method described previously (Costello et al., 2014 and (Gellner et al 2016b). Briefly, eight cigarettes were smoked through 35 ml of saline solution (35 ml puffs over 2 s, repeated every 30 s) and the final solution was adjusted to pH 7.2–7.4. The CSE solution was prepared fresh each day immediately before experimental testing in order to minimize differences resulting from differential stability of the constituents. All CSE doses were defined by the solutions nicotine content, which was analyzed by an outside facility (UCSF Clinical Pharmacology Laboratory).

Subjects

Male Sprague–Dawley rats were obtained from Charles River at postnatal (P) days 17 and 81. Adolescent rats remained with dam until weaning (P21). Animals were then group-housed throughout the experiment. All rats were

maintained on a 12-h light/dark cycle (lights on at 07:00 am) with food and water available *ad libitum*. No more than one animal per litter per experimental group was used to avoid potential confounds. All experimental procedures were in compliance with NIH guidelines and were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

Rats were minimally food-restricted beginning two days prior to operant conditioning to promote exploration of the operant chamber and aid in acquisition of the operant task. Adolescent and adult rats were fed 15–25 or 20–25 g of food, respectively, to maintain normal growth during self-administration testing. Food was given 15 min after each experimental session, and any remaining chow was removed an hour before the following day test session. Food maintenance continued until the end of the experiment. Growth curves for both adolescents and adults followed normal trajectories (data not shown).

Behavioral Studies

Apparatus

Animals were tested in plexiglass operant chambers (Med Associates, St Albans, VT), equipped with two levers. Responses at the reinforced (R) lever resulted in illumination of a cue light over the lever and activation of an externally mounted syringe pump that infused drug. During the infusion (5.6 s yielding 100 µl of solution) and timeout period (20 s) the cue light remained illuminated and the house light was turned off. Responses on the non-reinforced (NR) lever were recorded but had no consequences.

Food Training

Adolescent and adult rats, aged P25 and 85, respectively, were first trained to lever-press for food pellets (45-mg rodent purified diet; Bio-Serv, Frenchtown, NJ) under a fixed ratio 1 schedule with a 1 second timeout period (FR1TO1), followed by FR1TO10, and completed with FR1TO20. Rats progressed to the next timeout period when they earned at least 35 or 50 reinforcers (adolescents and adults, respectively) in the daily 30-minute session.

Surgery

Following successful acquisition of food responding, rats were anesthetized with equithesin (0.0035 ml/g body weight) and implanted with indwelling jugular vein catheters (Belluzzi et al 2005). During the 3-day recovery period, catheters were flushed daily with a heparinized saline solution to maintain patency. The day before initiation of self-administration, and at intervals thereafter, catheter patency was verified for rapid (5-10 s) anesthesia by infusing propofol (5 mg/kg, i.v.). Patency was tested at the end of each schedule and only animals showing rapid anesthesia were included in analyses.

Self-Administration

After recovery, adolescents and adults, aged P37 and 97, respectively, were allowed to self-administer a single dose of nicotine or CSE (0, 3.75, 7.5, 15, or 30 µg/kg/infusion nicotine content). Rats self-administered nicotine or CSE for 7 days at the FR1TO20 schedule, before transitioning to the FR2TO20 schedule

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for 2 days, and finishing with 3 days at the FR5TO20 schedule during daily 1-hour sessions.

Statistical Analyses

The average of the last 3 days of self-administration at the FR1 schedule (Day 5-7) and the FR5 schedule (Day 10-12) were analyzed separately with a 4-way ANOVA on Age x Drug x Dose x Lever with repeated measures on Lever. Significant main effects were further analyzed with Dunnett's, Bonferroni-corrected paired (levers) or unpaired (drug) t-test post hoc comparisons. Drug intake, calculated as the number of infusions per session multiplied by the dose of drug self-administered, was analyzed with a 4-way ANOVA on Intake x Age x Drug x Dose. Significant main effects were further analyzed with Bonferroni-corrected unpaired t-test post hoc comparisons. Non-reinforced (NR) responding data was analyzed with a 3-way ANOVA on NR responding x Age x Dose. Significant main effects were further analyzed with Dunnett's or Bonferroni-corrected unpaired t-test post hoc comparisons. Corrected reinforced responding data was analyzed with a 4-way ANOVA on R–NR responding x Age x Drug x Dose. Significant main effects were further analyzed with Bonferroni-corrected unpaired t-test post hoc comparisons.

Results

At the FR1 schedule of reinforcement, main effects of Levers $[F_{(1,165)}=352.285, p<0.0001]$, Age $[F_{(1,165)}=109.535, p<0.0001]$, Drug $[F_{(1,165)}=5.113, p<0.05]$, and Dose $[F_{(4,165)}=11.050, p<0.0001]$ were found.

Given the significant age difference, adolescents and adults were analyzed separately to further assess Drug and Dose effects (Figure 2.1). Adolescents showed significant effects of Levers [$F_{(1,81)}=167.009$, $p<0.0001$] and Dose [$F_{(4,81)}=8.646$, $p<0.0001$], but not Drug, indicating that the non-nicotine constituents do not enhance acquisition of self-administration behavior at this schedule (Figure 2.1a). Adolescents preferred the reinforced to the non-reinforced lever at all doses, including 0 ($p<0.05$ vs non-reinforced). Adolescents exhibited an inverted U dose-response curve for reinforced responding, with higher responses as compared to saline at the three lowest drug doses (Figure 2.1a). Non-reinforced responding at all drug doses was significantly higher than for saline.

At the FR1 schedule of reinforcement, adults showed significant effects of Levers [$F_{(1,84)}=273.606$, $p<0.0001$], Drug [$F_{(1,84)}=10.594$, $p<0.01$], and Dose [$F_{(4,84)}=3.252$, $p<0.05$]. Adults preferred the reinforced to the non-reinforced lever at all doses, including 0 ($p<0.05$ vs non-reinforced; Figure 2.1b). Whereas CSE exhibited a flat dose-response curve, there was enhanced reinforced responding for nicotine at the 7.5 dose as compared to saline ($p<0.05$ vs 0 dose). Animals responding for nicotine had significantly higher reinforced responding at the 7.5 and 30 μg/kg doses than for CSE with equivalent nicotine content ($p<0.05$ vs CSE).

When the schedule of reinforcement was increased to FR5, significant main effects of Levers [$F_{(1,165)}=192.43$, $p<0.0001$], Age [$F_{(1,165)}=31.903$, $p<0.0001$], Dose [$F_{(4,165)}=12.667$, $p<0.0001$] and a significant Drug*Dose

interaction [F(4,165)=3.013, p=0.020] were found. Adolescent and adult data were again split to analyze Drug and Dose effects. Adolescents showed significant effects of Levers [F(1,81)=100.335, p<0.0001] and Dose [F(4,81)=8.456, p<0.0001], but not Drug, indicating that the non-nicotine constituents do not enhance self-administration behavior in adolescents (Figure 2.2a). At this schedule, adolescent rats showed a preference for the reinforced lever at all doses, including 0 (p<0.05). In addition, adolescents showed enhanced reinforced and non-reinforced responding at the 3 highest doses compared to saline (p<0.001-0.0001).

At FR5 in adults there were significant effects of Levers [F(1,84)=92.011, p<0.0001] and Dose [F(4,84)=4.232, p=0.004], with a Lever*Drug interaction [F(1,84)=4.527, p=0.036]. As with adolescents, adults showed a preference for the reinforced lever at all doses, including 0 (p<0.05; Figure 2.2b). Although there was a significant Lever*Drug interaction, post-hoc analysis showed no significant differences in self-administration of the two drugs at any dose. However, reinforced responding was significantly higher than saline for CSE at the 15 µg/kg nicotine content dose, and for nicotine at the 30 µg/kg dose (p<0.05 vs 0 dose).

Drug intake is shown in Figure 2.3. At the FR1 schedule (Figure 2.3a), there were main effects of Age [F(1,165)=127.428, p<0.0001], Drug [F(1,165)=9.664, p<0.01], and Dose [F(4,165)=94.434, p<0.0001]. When data were split by Age, adolescents showed main effects of Dose [F(4,81)=51.385, p<0.0001] but not Drug, indicating that adolescents take similar amounts of CSE

and nicotine. Adolescents showed higher nicotine intake than adults at all drug doses (p<0.05). Adults displayed main effects of Drug [F(1,84)=31.087, p<0.0001] and Dose [F(4,84)=70727, p<0.0001], and had higher nicotine intake compared to CSE at the 7.5 and 30 µg/kg doses (p<0.05, p<0.01).

For drug intake at the FR5 schedule (Figure 2.3b), main effects of Age [F (1,165)=13.761, p<0.0001], Drug [F(1,165)=4.960, p=0.027], and Dose [F(4,165)=65.152, p<0.0001] were found. When data were split by Age, adolescents showed significant effects of Dose [F(4,81)=41.470, p<0.0001] with a Drug*Dose interaction [F(4,81)=3.838, p=0.007]. Post hoc analysis revealed that adolescents had higher nicotine intake as compared to CSE at the 30 µg/kg dose (p<0.05). Adults showed significant effects of Drug [F(1,84)=3.946, p=0.050] and Dose [F(4,81)=23.968, p<0.0001], but further analysis did not reveal any significant drug differences.

To examine if the increase in drug intake during adolescence at the FR1 schedule was due to non-specific activity alone, non-reinforced responding was analyzed separately (Figure 2.4). At the FR1 schedule, there were main effects of Age [F(1,175)=73.496, p<0.0001] and Dose [F(4,175)=5.069, p<0.01], but not Drug. Adolescents, but not adults, showed a drug-related increase in non-reinforced responding during the FR1 schedule (Figure 2.4a). Non-reinforced lever pressing on the FR5 schedule of reinforcement also showed main effects of Age [F(1,175)=53.235, p<0.0001] and Dose [F(4,175)=6.878, p<0.0001], but not Drug. Again, adolescents, but not adults, showed a drug-related increase in non-specific activity (Figure 2.4b).

To correct for differences in non-reinforced responding and allow for an accurate age comparison, non-reinforced responding was subtracted from reinforced responding for each animal (Figure 2.5). At the FR1 schedule (Figure 2.5a), main effects of Age [$F_{(1,165)}=25.194$, $p<0.0001$], Drug [$F_{(1,165)}=4.580$, $p<0.05$], and Dose [$F_{(4,165)}=8.882$, $p<0.0001$] were found. An Age comparison showed that adolescent responding for both drugs was significantly higher than that of adults at the 3.75 dose ($p<0.001$). Adolescents also showed main effects of Dose [$F_{(4,81)}=6.571$, $p<0.0001$] but not Drug, indicating that adolescents self-administer nicotine and CSE equally at all doses. Adults had main effects of Drug [$F_{(1,84)}=14.837$, $p<0.0001$] and Dose [$F_{(4,84)}=4.470$, $p<0.001$], with post hoc analysis showing significantly lower adult responding for CSE than for nicotine at the three highest doses ($p<0.05$). At the FR5 schedule of reinforcement, main effects of Dose [$F_{(4,165)}=7.420$, $p<0.0001$] but not Age [$F_{(1,165)}=2.351$, $p=0.127$] or Drug [$F_{(1,165)}=3.204$, $p=0.075$] were found. Thus, adolescent and adult male rats show similar self-administration behavior on the FR5 schedule of reinforcement when corrected for non-reinforced responding (Figure 2.5b).

Discussion

The present study focused on understanding whether aqueous constituents of tobacco smoke influence acquisition of nicotine self-administration in adolescent and adult male rats. As has been shown previously in adults (Costello et al 2014), we now demonstrate that adolescents also acquire self-administration of CSE. Nicotine and CSE similarly increased non-reinforced responding in adolescents at both FR1 and FR5 schedules of reinforcement,

leading to enhanced overall drug intake as compared to adults. When data were corrected for age-dependent alterations in non-reinforced responding, adolescents were found to be more sensitive to low doses of nicotine and CSE than were adults at the low, FR1 reinforcement schedule. There were no differences in adolescent responding for the two drugs at this schedule, whereas adults had fewer responses for CSE than for nicotine at equivalent doses. When the task was made harder by increasing to a FR5 reinforcement schedule, animals' dose-dependently self-administered both nicotine and CSE, but no drug or age differences were observed.

Methodological issues

Traditionally, rat self-administration studies have examined the effects of nicotine alone in adults (Corrigall and Coen 1989; Donny et al 1995). However, more recent studies have begun to examine the effects of the non-nicotine constituents present in cigarette smoke. Individual constituents, such as minor alkaloids, monoamine oxidase inhibitors and acetaldehyde, have been shown to enhance nicotine self-administration (Belluzzi et al 2005; Guillem et al 2005; Villégier et al 2006; Hall et al 2014; Arnold et al 2014). However, these studies do not examine the combined effects of tobacco smoke constituents. Smoke extracts have been shown to contain many combustion products that are not present in tobacco extracts (Bates et al 1999; Seeman et al 2002; Brennan et al 2014) and potentially provide a better model of tobacco dependence.

We have previously shown that adult male rats will self-administer aqueous CSE, and that this was more potent than equivalent doses of nicotine

alone (Costello et al 2014). In the present study, in contrast to our earlier finding, we did not find CSE to be more potent than nicotine in adults; indeed, at a low reinforcement schedule it was not self-administered more than saline. At the FR5 schedule used previously by our group (Costello et al 2014), we found both CSE and nicotine to be self-administered by adults but with no significant differences between drug groups.

This discrepancy may reflect methodological differences between the two studies, with experimental modifications being introduced in the current study to accommodate the needs of adolescent rats. In our earlier study, two experimental approaches were used, both of which were different from those used here. The first was to conduct drug acquisition training at an FR1 schedule using nose pokes and no prior food training. This approach was determined to be unsuitable for use in adolescents because of their high non-reinforced responding on nose pokes. The second was to food train on levers to an FR5, not FR1, schedule, and then use the same training dose of drug for all animals followed by a within-subjects dose response analysis. In the present study, prolonged food training at FR1 was necessary for adolescents, and was not extended to FR5 because of constraints in the duration of this developmental stage. Instead, all animals were food trained to FR1 then switched to different doses of nicotine or CSE in a between-subjects design, similar to that employed by (Donny et al 1998). Following stable responding at FR1, animals were then escalated to drug responding at FR5, an approach that worked for both ages.

Age-differences in drug sensitivity

We have previously shown that nicotine stimulates locomotor activity in adolescent rats, while reducing it in adults (Cao et al 2010). Consistent with this observation, both nicotine and CSE increased non-reinforced responding, a measure of activity, in adolescents but not adults. This hyperactivity resulted in substantially higher nicotine intake in the younger animals that self-administered either nicotine or CSE. When this higher activity level was corrected for, by subtracting non-reinforced lever presses, adolescent rats worked harder than adults for the lowest dose of drug (3.75 μg/kg/infusion nicotine content) on the FR1 reinforcement schedule. Whereas adolescent rats self-administered similar amounts of CSE and nicotine on this schedule, adult rats self-administered more nicotine than CSE at the higher doses. However, both age and drug differences were eliminated when the task was made harder by increasing the reinforcement schedule to FR5. Thus, the differences observed at FR1 were not robust. Our findings are consistent with other studies that have shown age differences in responding for low doses of nicotine at differing schedules of reinforcement (Shram et al 2008b; Schassburger et al 2016). However, in contrast to these other studies, we have found adolescent rats to be more sensitive to the reinforcing effect of low doses of drug at the FR1 schedule.

Clinical implications

Our current findings demonstrate that nicotine alone is as reinforcing to male adolescent rats as nicotine combined with other tobacco smoke constituents. This finding is important given recent epidemiological observations

of a switch in teenagers' initial preference from smoking conventional cigarettes to e-cigarettes (Singh et al 2016). Our preclinical data are consistent with clinical observations that suggest that nicotine delivered through e-cigarettes is reinforcing and may encourage subsequent conventional cigarette use in teen smokers (Dutra and Glantz 2014). It should be noted that initial acquisition, as measured here, is only one measure by which the addictive properties of nicotine alone can be compared with cigarette smoke. Other measures, including withdrawal and craving or reinstatement, may show significant differences in the effects of nicotine alone or with other tobacco smoke constituents. A recent study has noted that passive exposure to the smoke of e-cigarettes resulted in lower precipitated withdrawal in mice than exposure to smoke from conventional cigarettes (Ponzoni et al 2015). Having established a self-administration model in adolescent rats, we can in future determine whether extinction and reinstatement are differentially impacted by presence of tobacco smoke constituents in CSE.
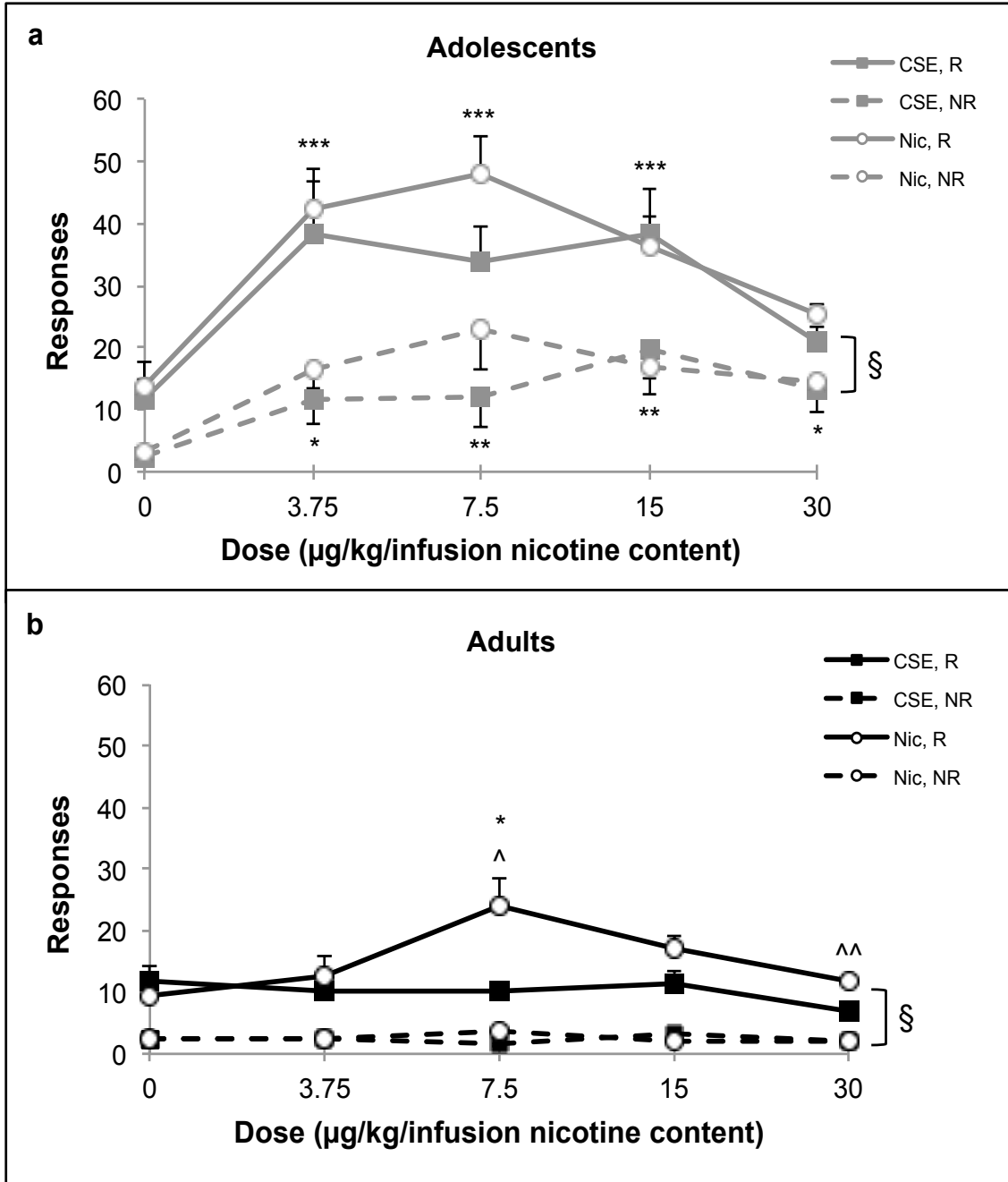
Fig. 2.1. Adolescent and adult self-administration of CSE and nicotine at the FR1 schedule. Data shown are an average of the last three days of self-administration. Both adolescents (**a**) and adults (**b**) preferred the reinforced lever at all doses (§p<0.05). Significantly different from saline, *p<0.05, **p<0.01, ***p<0.0001; significantly different from CSE, ^p<0.05, ^^p<0.01. n = 8-12 per group
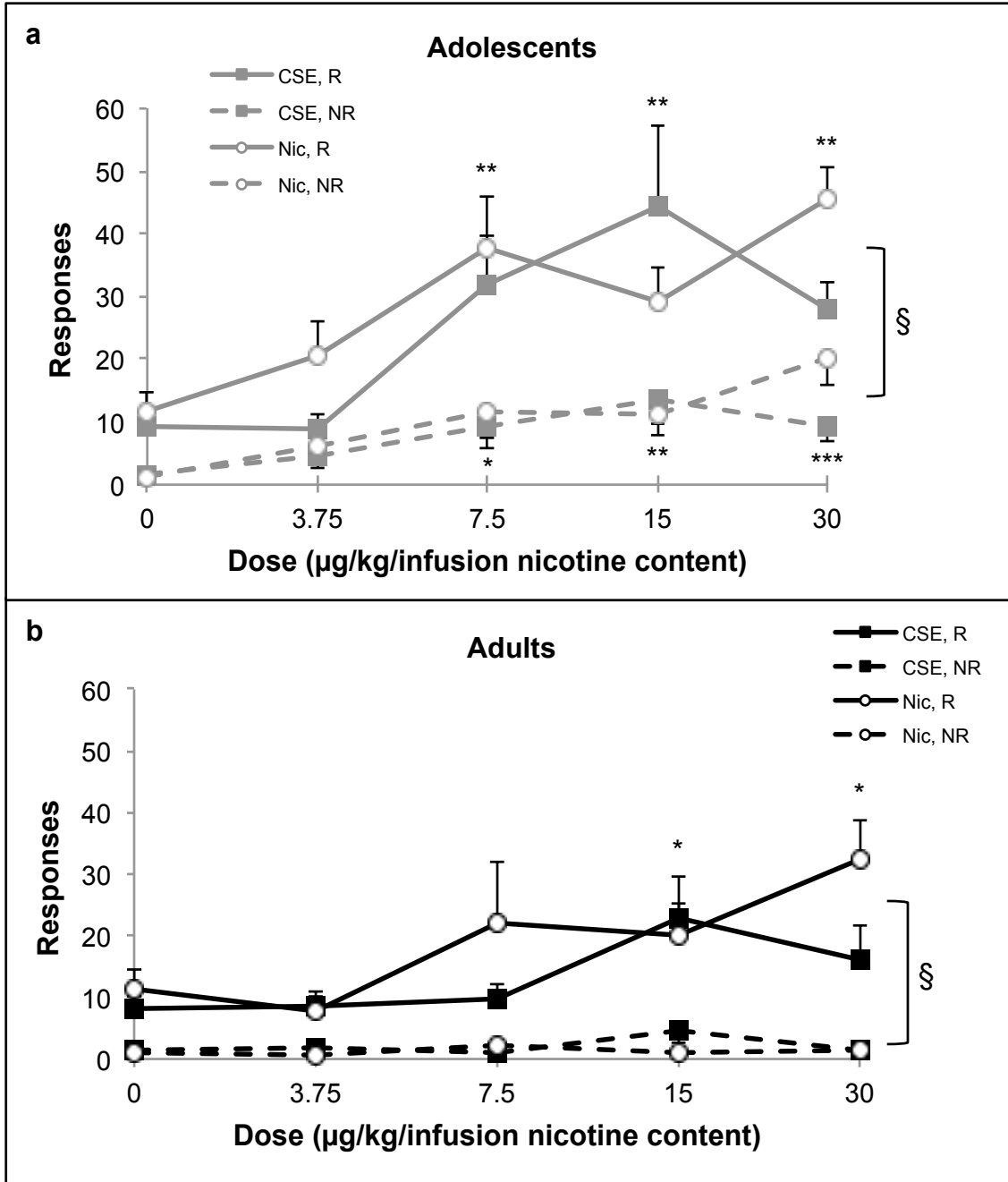
Fig. 2.2. Adolescent and adult self-administration of CSE and nicotine at the FR5 schedule. Data shown are an average of the last three days of self-administration. Both adolescents (**a**) and adults (**b**) preferred the reinforced lever at all doses (§p<0.05). Significantly different from saline, *p<0.05, **p<0.01, ***p<0.0001. n = 8-12 per group
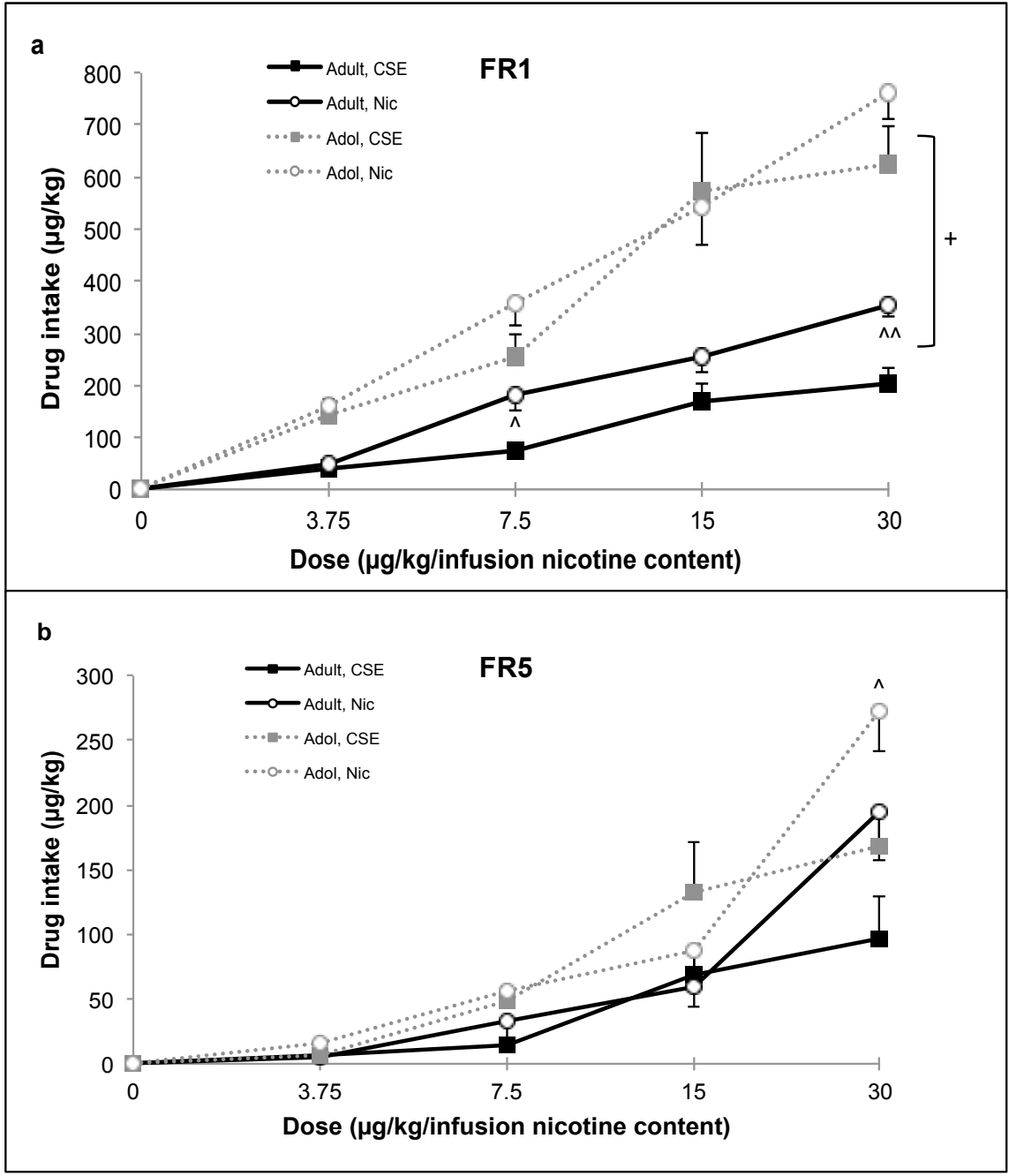
Fig. 2.3. Adolescents take more drug than adults at the FR1 schedule. Intake at the FR1 (**a**) and FR5 (**b**) is calculated as the number of infusions per session multiplied by the self-administered dose. Adolescents significantly different from adults, +p<0.05; nicotine significantly different from CSE, ^^p<0.01, ^p<0.05. n = 8-12 per group
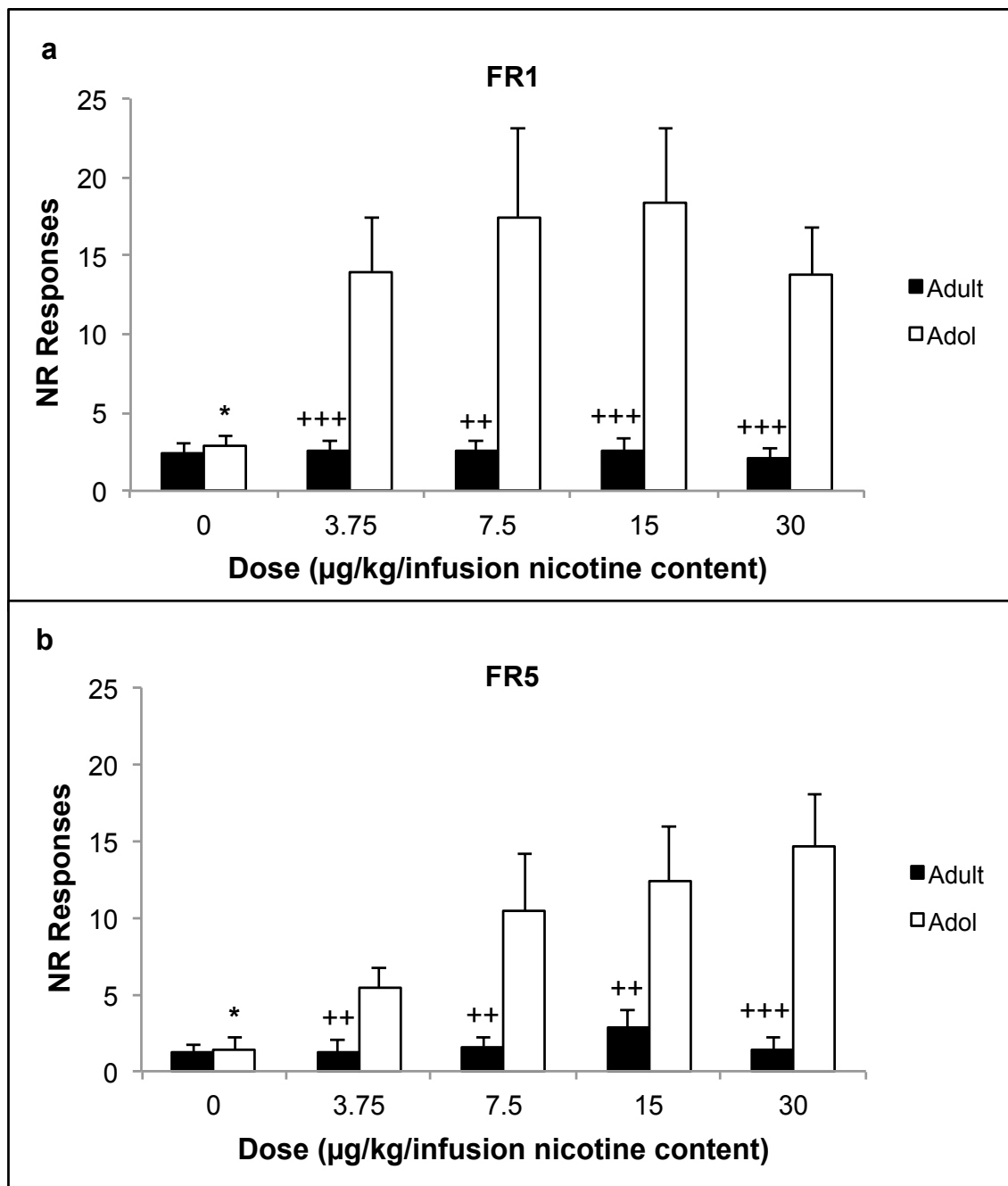
Fig. 2.4. Adolescent rats show drug-induced increases in non-reinforced responding. Due to a lack of drug effect, CSE and nicotine non-reinforced responding were combined at the (**a**) FR1 and (**b**) FR5 schedules. *p<0.05 vs all other doses; +++p<0.0001, ++p<0.01 vs adults. n=8-12 per group

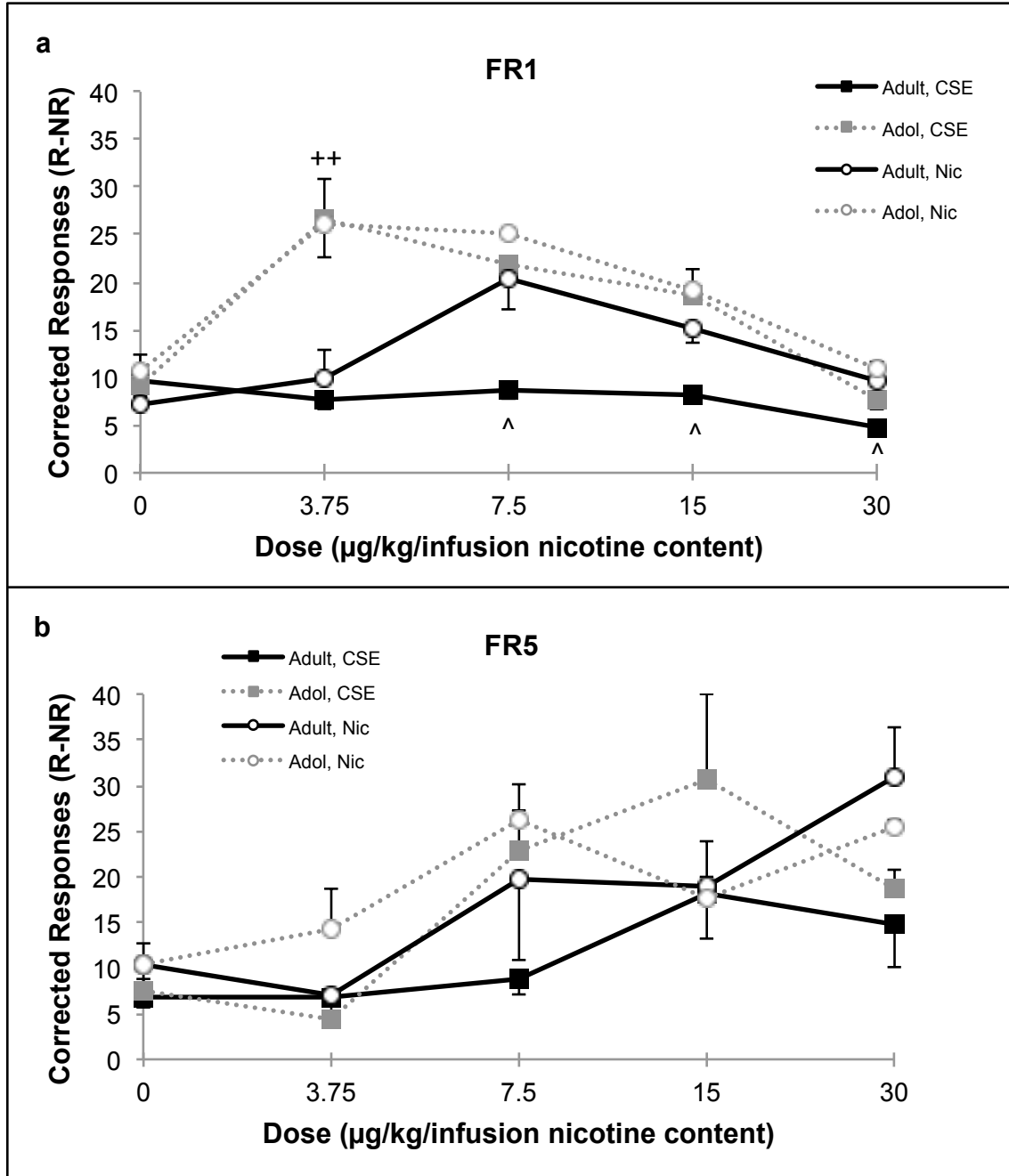**Fig. 2.5. Adolescents self-administer more low-dose nicotine than adults when differences in non-reinforced responses are corrected for.** (**a**) Adolescents self-administer more drug than adults on the FR1 schedule (++p<0.001). Adults self-administer more nicotine than CSE at the three highest doses (^p<0.05). (**b**) Adolescent and adult rats behave similarly on the FR5 schedule. n=8-12/group

Chapter 3: Tobacco Smoke Constituents Enhance Reinstatement of Nicotine-Seeking Behavior

Introduction

Tobacco addiction is a chronic relapsing brain disorder (Miller and Chappel 1991; Leshner 1999; Lubman et al 2004) in which users continue to smoke despite negative health consequences. Initiation of smoking occurs during adolescence, with 9 out of 10 smokers trying their first cigarette by the age of 18 (Center for Disease Control and Prevention (CDC) 2014). Of these adolescents, nearly 50% try to quit (Kann et al 2016). Unfortunately 3 out of 4 smokers continue smoking into adulthood (Surgeon General 2012). Of the teenagers who are successful in their attempt to quit, 60-90% end up relapsing in the first year of cessation (Diemert et al 2013); demonstrating the need for a better understanding of adolescent relapse to smoking.

To study relapse to smoking, animal models employ the use of triggers, such as stress, cues, or re-exposure to the drug. These triggers are known to cause relapse to smoking in humans and have also been shown to readily reinstate drug-seeking behavior in animals (Shaham et al 2003). Animal models of relapse to date have focused on adult reinstatement of drug-seeking behavior, although clinical studies suggest that those who start smoking and try to quit as adolescents are more likely to relapse than adults (Royal College of Physicians of London. Tobacco Advisory Group 2000). Anker and Carroll (2010) demonstrated that adolescent rats, that had previously self-administered cocaine, displayed enhanced reinstatement to stress and re-exposure to the drug. In

contrast, Shram et al. (2008a) showed that the age of onset of self-administration plays no role in the degree of reinstatement to nicotine-seeking behavior after re-exposure to drug. Although these studies begin to investigate the role of age in relapse, our understanding of certain triggers such as stress, cues, and the combination of stress and cues have yet to be investigated during adolescence.

Of the triggers that lead to relapse, stress has been identified as a major cause of relapse in humans (Buczkowski et al 2014). To study stress-induced relapse using animal models of tobacco dependence, researchers employ the use of footshock, a physiological stressor, or yohimbine, a pharmacological stressor, to induce stress. The α2 adrenergic receptor antagonist, yohimbine, has been shown to produce more reliable stress-induced reinstatement of nicotine-seeking behavior as compared to footshock (Lê et al 2005; Marinelli et al 2007). Our lab has shown that the presence of tobacco smoke constituents during self-administration enhances yohimbine-induced reinstatement as compared to adult male rats that self-administered nicotine alone (Costello et al., 2014). I have previously shown that the tobacco smoke constituents do not influence acquisition of adolescent self-administration Chapter 2 (Gellner et al 2016a). However, the influence of tobacco smoke constituents on yohimbine-induced reinstatement of CSE- or nicotine-seeking in adolescents is unknown. Based on these studies, I hypothesized that adolescents would show enhanced stress-induced reinstatement of CSE- or nicotine-seeking compared to adults; with no effect of tobacco smoke constituents on adolescent behavior.

In order to investigate the role of age and tobacco smoke constituents in relapse to smoking I have now compared yohimbine-induced reinstatement of CSE- or nicotine-seeking behavior in adolescent and adult male rats. As human adolescents are just as susceptible to relapse as adults (Karpinski et al 2010), it is vital to understand how stress, an extremely important trigger for relapse, influences both adolescents and adults, and whether prior exposure to tobacco smoke constituents influences this.

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 by bubbling smoke from commercial cigarettes (Camel unfiltered, RJ Reynolds) through sterile saline (Costello et al., 2014; (Gellner et al 2016b). Briefly, eight cigarettes were smoked through 35 ml of saline solution (35 ml puffs over 2 s, repeated every 30 s) and the final solution was adjusted to pH 7.2–7.4. The CSE solution was prepared fresh each day immediately before experimental testing in order to minimize differences resulting from differential stability of the constituents. All CSE doses were defined by the solution's nicotine content, which was analyzed by GC-MS (Finnigan Trace MS with Trace GC 2000 series, UCI Mass Spectrometry Facility). Yohimbine hydrochloride (Sigma-Aldrich) was dissolved in double distilled water.

Subjects

Male Sprague–Dawley rats were obtained from Charles River at postnatal (P) days 17 and 81. Adolescent rats remained with dam until weaning (P21). Animals were then group-housed throughout the experiment. All rats were maintained on a 12-h light/dark cycle (lights on at 07:00 am) with food and water available ad libitum. No more than one animal per litter per experimental group was used to avoid potential confounds. All experimental procedures were in compliance with NIH guidelines and were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

Rats were minimally food-restricted beginning two days prior to operant conditioning to promote exploration of the operant chamber and aid in acquisition of the operant task. Adolescent and adult rats were fed 15–25 or 20–25 g of food, respectively, to maintain normal growth during self-administration testing. Food was given 15 min after each experimental session, and any remaining chow was removed an hour before the following day test session. Food maintenance continued until the end of the experiment. Growth curves for both adolescents and adults followed normal trajectories (data not shown).

Dose-response of yohimbine

Naïve animals (P34 and 84) were handled for 3 days to minimize stress before being injected with yohimbine (0, 0.3, 0.8, or 2.5 mg/kg, i.p.). Sixty minutes later trunk blood was collected for corticosterone analysis using an ImmuChem Double Antibody Corticosterone 125I kit (MP Biomedicals, LLC).

Behavioral Studies

Apparatus

Animals were tested in plexiglass operant chambers (Med Associates, St Albans, VT), equipped with two levers. Responses at the reinforced (R) lever resulted in illumination of a cue light over the lever and activation of an externally mounted syringe pump that infused drug. During the infusion (5.6 s yielding 100 µl of solution) and timeout period (20 s) the cue light remained illuminated and the house light was turned off. Responses on the non-reinforced (NR) lever were recorded but had no consequences.

Food Training

Adolescent and adult rats, aged P25 and 85, respectively, were first trained to lever-press for food pellets (45 mg rodent purified diet; Bio-Serv, Frenchtown, NJ) under a fixed ratio 1 schedule with a 1 second timeout period (FR1TO1), followed by FR1TO10, FR2TO20, and completed with FR5TO20. Rats progressed to the next schedule when they earned at least 35 or 50 reinforcers (adolescents and adults, respectively) in two 30-minute sessions per day.

Surgery

Following successful acquisition of food responding, rats were anesthetized with equithesin (0.0035 ml/g body weight) and implanted with indwelling jugular vein catheters (Belluzzi et al 2005). During the 2-3-day recovery period, catheters were flushed daily with a heparinized saline solution to

maintain patency. The day before initiation of self-administration, and at intervals

thereafter, catheter patency was verified for rapid (5-10 s) anesthesia by infusing

propofol (5 mg/kg, i.v.). Patency was tested at the end of each schedule and only

animals showing rapid anesthesia were included in analyses.

Reinstatement of drug seeking

After recovery, adolescents and adults, aged P37 and 97, respectively,

were allowed to self-administer nicotine or CSE (15 µg/kg/infusion nicotine

content) at the FR5TO20 schedule during daily 1-hour sessions. Rats self-

administered nicotine or CSE for 10 days or until stable criteria were met (R

within 20% of the mean over 3 days; R >= 2 × NR responses; R >= 6). After

reaching stable responding, extinction testing (house light on, animals not

connected to the infusion tubing and responses on the levers had no

consequence) began and lasted for 5 days or until extinction criteria were met

(R<20% of last FR5 responding). When animals met extinction criteria,

reinstatement testing began.

Nicotine- or CSE-seeking was reinstated using three conditions; stress

only (2.5 mg/kg yohimbine, i.p 30 min before session), cues only (reintroduction

of cue light), or stress + cues (combination of the previous conditions based on

Feltenstein et al., 2012) with a minimum of 2 days extinction between each test.

Conditions were given in a within counterbalanced design. Animals then

underwent a final test in which they were injected with yohimbine (0 or 2.5 mg/kg,

i.p.), placed into the operant chamber, with levers removed to control for activity

differences in adolescents and adults, for 30 minutes before being decapitated. Whole blood was collected to analyze corticosterone levels using an ImmuChem Double Antibody Corticosterone 125I kit (MP Biomedicals, LLC).

Statistical Analyses

Yohimbine dose response data were analyzed using a 3-way ANOVA on Corticosterone Level x Age x Dose. Significant main or interaction effects were further analyzed with a 2-way ANOVA for each Age separately on Corticosterone Level x Dose with Dunnett's adjusted post hoc comparisons. Self-administration at the FR5 schedule was analyzed using a 4-way ANOVA on R/NR x Day x Age x Drug with repeated measures on R/NR and Day. Significant main or interaction effects were further analyzed with a 2-way ANOVA for each Age or Day separately on R/NR x Day or R/NR x Age with Bonferroni-corrected paired (R/NR and Day) or unpaired (Age) t-test post hoc comparisons. Extinction data were analyzed using a 4-way ANOVA on Percent of FR5 baseline x Day x Age x Drug with repeated measures on Day. Significant main or interaction effects were further analyzed with a 2-way ANOVA for each Age or Day separately on Percent of FR5 baseline x Day or Percent of FR5 baseline x Age with Bonferroni-corrected paired (Day) or unpaired (Age) t-test post hoc comparisons. Reinstatement data were analyzed using a 4-way ANOVA on Percent of FR5 baseline x Condition x Age x Drug with repeated measures on Condition. Significant main or interaction effects were further analyzed with a 2-way ANOVA for each Drug or Condition separately on Percent of FR5 baseline x Condition or

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Percent of FR5 baseline x Drug with Bonferroni-corrected paired (Condition) or unpaired (Drug) t-test post hoc comparisons.

Results

Yohimbine-induced corticosterone release

A dose response analysis of yohimbine-induced corticosterone release was conducted in adolescent and adult male rats (Figure 3.1). Overall significant main effects of Dose [$F_{(3,60)}=69.130$, $p<0.0001$] and an interaction between Age and Dose [$F_{(3,60)}=7.239$, $p<0.0001$] were found. When each age was examined separately, both adolescents and adults displayed significant main effects of Dose [$F_{(3,33)}=24.024$, $p<0.0001$ and $F_{(3,27)}=51.178$, $p<0.0001$, respectively]. Basal corticosterone levels did not show an age difference. However, whereas adolescents showed a monotonic increase in yohimbine-induced corticosterone levels, adults showed a U-shaped function. In adolescents, yohimbine significantly increased plasma corticosterone levels at 0.8 and 2.5 mg/kg doses ($p=0.029$ and $p<0.0001$, respectively), whereas adults showed significant increases at 0.3 and 2.5 mg/kg doses ($p=0.048$ and $p<0.0001$, respectively). Plasma corticosterone levels in adolescents were significantly higher than adults following 0.8 mg/kg yohimbine ($p=0.024$), but significantly lower at the 0.3 and 2.5 mg/kg doses ($p=0.048$ and 0.013, respectively). Given that 2.5 mg/kg yohimbine induced significant corticosterone secretion in both adolescents and adults, this dose was chosen for use in subsequent experiments.

Initial self-administration

Both adolescents and adults were found to achieve stable self-administration of nicotine and CSE over an initial 10-day period (Figure 3.2). Overall main effects of Days $[F(9,459)=7.118, p<0.0001]$ and Age $[F(1,51)=25.332, p<0.0001]$, with an interaction between Days x Age $[F(9,459)=4.326, p=0.0001]$ were found. Since no overall or interaction effects of Drug were found, nicotine and CSE data were combined for the analysis (Figure 3.2). Post-hoc analysis showed that adolescents self-administered more drug and responded more on the NR lever compared to adults on all days except day 1 ($p<0.005$, Figure 3.2).

Extinction and reinstatement

Following stable self-administration, drug-seeking behavior was extinguished by removal of drug and cues (Figure 3.3). An overall main effect of Days $[F(5,255)=244.298, p<0.0001]$ and an interaction between Days x Age $[F(5,255)=11.845, p<0.0001]$ were found. Since no significant overall or interaction effects of Drug were found, nicotine and CSE data were combined (Figure 3.3). Post-hoc analysis showed that adolescents extinguished significantly faster than adults on Day 1 of extinction ($p<0.0001$), but less than adults on Days 3 and 5 ($p=0.037$ and $p=0.002$, respectively).

Following extinction, animals were triggered to reinstate drug-seeking behavior with yohimbine stress, cues, or the combination of stress and cues (Figure 3.4). Overall main effects of Condition $[F(3,153)=37.399, p<0.0001]$ and Drug $[F(1,51)=6.437, p=0.014]$ were found. Since no overall or interaction effects

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of Age were found, adolescent and adult data were combined (Figure 3.4). Both stress and the combination of stress and cues induced reinstatement of both CSE- and nicotine-seeking behavior ($p<0.0001$). Animals that had self-administered CSE responded more for cues and the combination of stress + cues compared to those that had worked for nicotine ($p=0.044$ and $p=0.020$, respectively; Figure 3.4).

HPA axis activation after stress-induced reinstatement

Further analysis of yohimbine-induced corticosterone secretion was done at the end of the study to examine a possible role of HPA axis activation in stress-induced reinstatement (Figure 3.5). There were main effects of Treatment [$F(1,27)=57.927$, $p<0.0001$], but not of Age [$F(1,27)=0.559$, $p=0.461$] or Drug [$F(1,27)= 0.556$, $p=0.462$], nor were there any interactions between Age, Drug, and Treatment [$F(1,27)=0$, $p=0.993$]. Yohimbine significantly enhanced corticosterone levels across all groups ($p<0.0001$, Figure 3.5).

Discussion

Adolescents who try to quit are more susceptible to relapse than adults (Royal College of Physicians of London. Tobacco Advisory Group 2000), yet the preclinical research field still focuses on studying adult relapse (LeSage et al 2004; Feltenstein et al 2012; Le Foll et al 2012; Liu 2014). In order to understand why adolescents may be more susceptible to relapse, the present study focused on understanding the role of age and tobacco smoke constituents in stress- and cue-induced reinstatement of nicotine-seeking behavior. As this is the first study

to compare adolescent and adult stress-induced reinstatement of nicotine seeking behavior, a dose response of yohimbine was done to find an optimum dose at which both adolescents and adults showed yohimbine-induced corticosterone release. Adolescents and adults showed distinct dose-response patterns of yohimbine-induced corticosterone secretion. Since the highest (2.5 mg/kg) dose examined induced corticosterone secretion in both adolescents and adults, this dose was chosen for subsequent analysis of stress-induced reinstatement.

Previous studies done in our lab have shown that stress-induced reinstatement is enhanced in adults that have self-administered CSE, as compared to those self-administering nicotine alone (Costello et al 2014). In the present study, I now show that there are no age differences in reinstatement responding, although drug differences exist, with those animals that had self-administered CSE responding more to cues alone and showing higher stress+cue-induced reinstatement compared to those animals that self-administered nicotine alone.

Tobacco smoke constituent effects on drug-seeking behavior

Previous studies have shown that cues can induce reinstatement of nicotine-seeking behavior in adult animals (Costello et al 2014; Liu et al 2008; Liu et al 2007). I now show that regardless of age, animals do not reinstate nicotine-seeking behavior after the presentation of cues. My experimental design was such that animals would experience the condition in a random order and experiencing stress prior to cues may decrease their subsequent seeking

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behavior after presentation of cues. This was not the case, as statistical analysis did not reveal an effect of order in which cues were presented on levels of cue-induced reinstatement (unpublished data). However, the differences observed may be due to methodological differences in which other studies used multiple cues (tone and light), substituted saline for nicotine during reinstatement, or only studied adult reinstatement.

Previous studies investigating the role of age in reinstatement responding have shown that adolescents reinstate cocaine-seeking behavior to a greater extent than adults (Anker and Carroll 2010). I now show that adolescents reinstate nicotine-seeking behavior to a similar extent as adults. These studies suggest that different mechanisms may mediate cocaine and nicotine reinstatement. Previous studies have shown that adults displayed enhanced responding during reinstatement of CSE-seeking behavior (Costello et al 2014). I now show that there is greater reinstatement – a possible model of craving – in both adolescent and adult animals that previously worked for CSE. My results suggest that regardless of age, the non-nicotine tobacco smoke constituents present in CSE enhance reinstatement of drug-seeking behavior.

Age-differences in self-administration and extinction

In line with my current results, previous studies have shown that adolescents take more drug than adults (Levin et al 2003; Chen et al 2007; Levin et al 2007; Gellner et al 2016a). Furthermore, upon initiation of extinction, adults were shown to maintain their initial responding more than adolescents. These results are in agreement with previous studies in which adults were shown to

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have significantly higher responding than adolescents during the first few days of extinction (Shram et al 2008a). In contrast to Shram et al. (2008a), however, I found that adolescents extinguished more slowly on subsequent days. Previous studies from our lab have shown that prior CSE self-administration enhanced responding on Day 1 of extinction (Costello et al 2014). I now show that the presence of non-nicotine tobacco smoke constituents do not enhance self-administration or extinction in adolescents and adults. These results may indicate that age is more important than the presence of tobacco smoke constituents during self-administration and extinction.

HPA axis activation after stress-induced reinstatement

Previous studies have implicated two main mechanisms underlying stress, the extrahypothalamic CRF system, which is mediated by CRF receptors in the brain, or activation of the HPA axis (Stephens and Wand 2012). In order to elucidate the role of the HPA axis in stress-induced reinstatement, corticosterone release was investigated. Previous studies indicate that patterns of corticosterone release are heavily dependent on length of stressor, time analyzed after the stressor, and the age of the animal (Romeo et al 2006). To my knowledge there are no studies investigating corticosterone release after yohimbine exposure in both adolescents and adults. In order to fill that gap, preliminary studies using naïve animals revealed that yohimbine induced distinct patterns on corticosterone release in the two age groups. However, following the completion of the reinstatement study, yohimbine enhanced corticosterone release to the same degree in all treatment groups. These results suggest that

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self-administration of drug and subsequent reinstatement of drug-seeking behavior may alter corticosterone release in which both adolescents and adults display similar HPA axis activation after yohimbine exposure. Furthermore, my results suggest that the tobacco smoke constituents present in CSE do not enhance this activation of the HPA axis. Further studies would need to be performed to elucidate whether drug-related cues would impact this finding, and to evaluate the potential role of the extrahypothalamic CRF system.

Conclusion

This study provides further experimental evidence implicating the importance of the tobacco smoke constituents role in animal models of tobacco dependence. Our laboratory has previously shown that CSE enhances stress-induced reinstatement in adults (Costello et al 2014). I now show that this effect is not due to enhanced activation of the HPA axis. I also show that there are no age differences in this effect, even though yohimbine induced differing dose-response profiles of corticosterone release in naïve adolescent and adult rats. My findings add to a growing body of literature indicating the importance of age and tobacco smoke constituents in animal models of tobacco dependence.
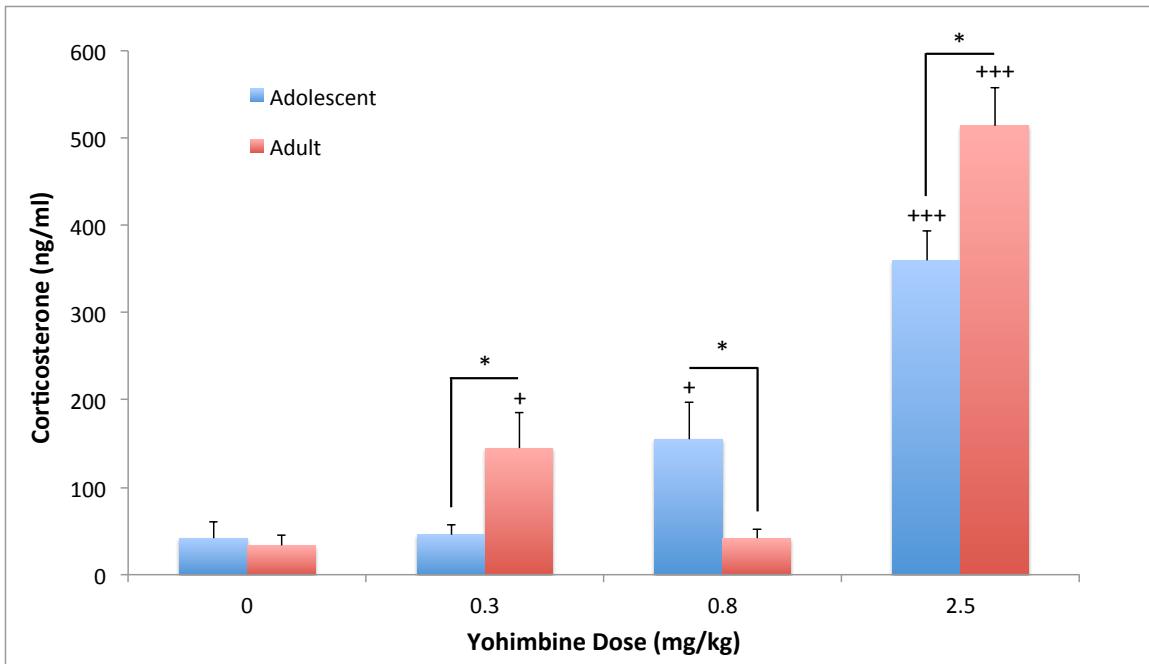
Figure 3.1. Age differences in yohimbine-induced plasma corticosterone secretion. The bars represent mean (±SEM) corticosterone levels after yohimbine injections for adolescent (blue bars) and adult (red bars) rats. The α2 AR antagonist, yohimbine (0, 0.3, 0.8 and 2.5 mg/kg, i.p.) was given 1 hour before trunk blood was collected and analyzed for corticosterone levels.
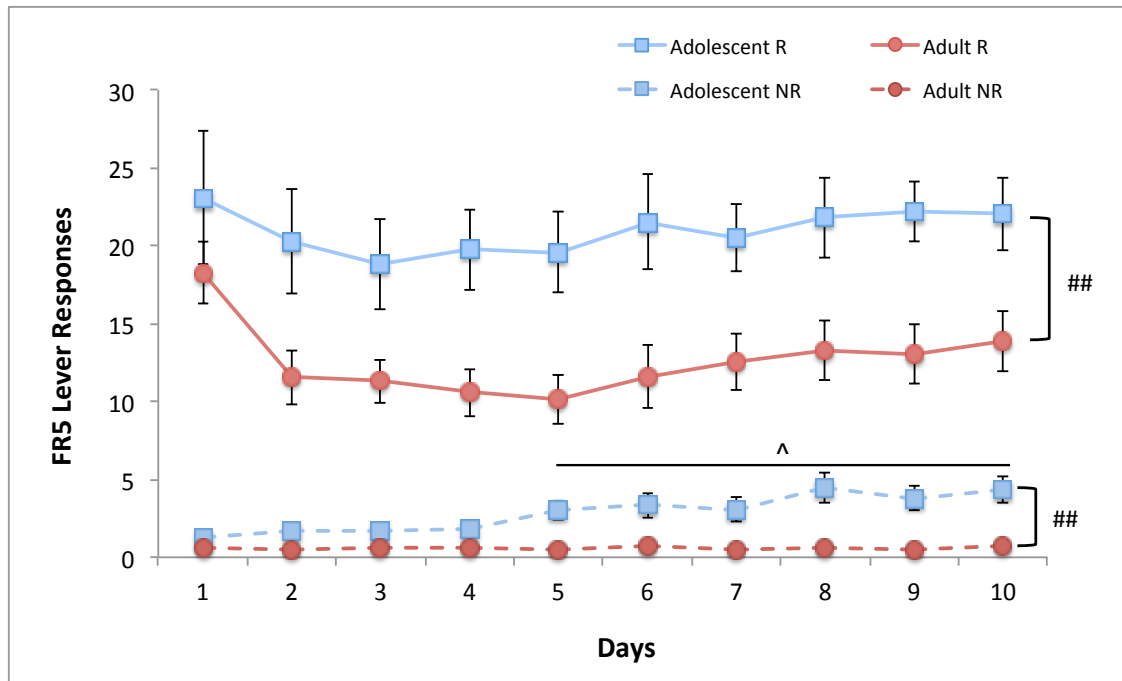+ $p<0.05$, +++ $p<0.0001$ vs. 0 dose; * $p<0.05$ vs. adult. n=7-10 per group.

Figure 3.2. Age, but not drug, differences in acquisition of self-administration. The lines represent mean (±SEM) responses leading to an infusion (R) of drug or not (NR) for adolescent (blue lines) and adult (red lines) rats. CSE and nicotine data were combined as no drug differences were seen. ## $p<0.005$ vs. adult, on all days except Day 1. ^ $p<0.05$ vs. Day 1 NR in adolescents only. n=12-15 per group.

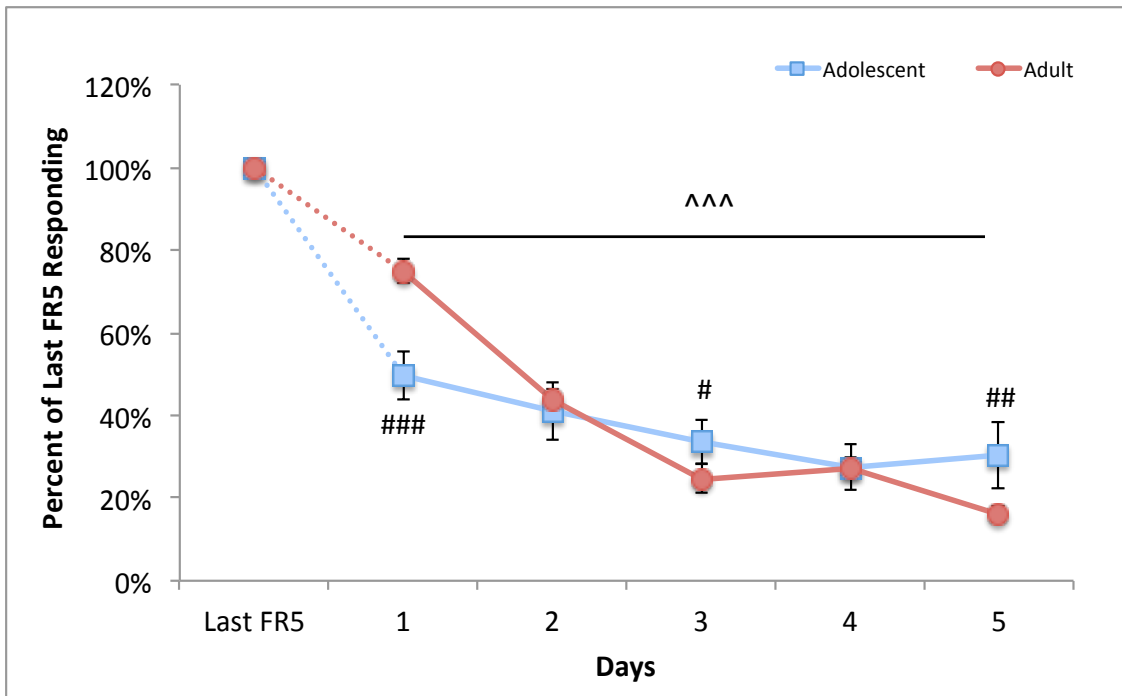**Figure 3.3. Age, but not drug, differences in extinction of responding.**
The lines represent mean (±SEM) responding as a percentage of their last
self-administration day for adolescent (blue) and adult (red) rats. Since there
were no drug differences, CSE and nicotine data were combined. ^^^
$p < 0.0001$ vs. Last FR5. ### $p < 0.0001$, ## $p < 0.01$, # $p < 0.05$ vs. adult. n=12-15
per group.

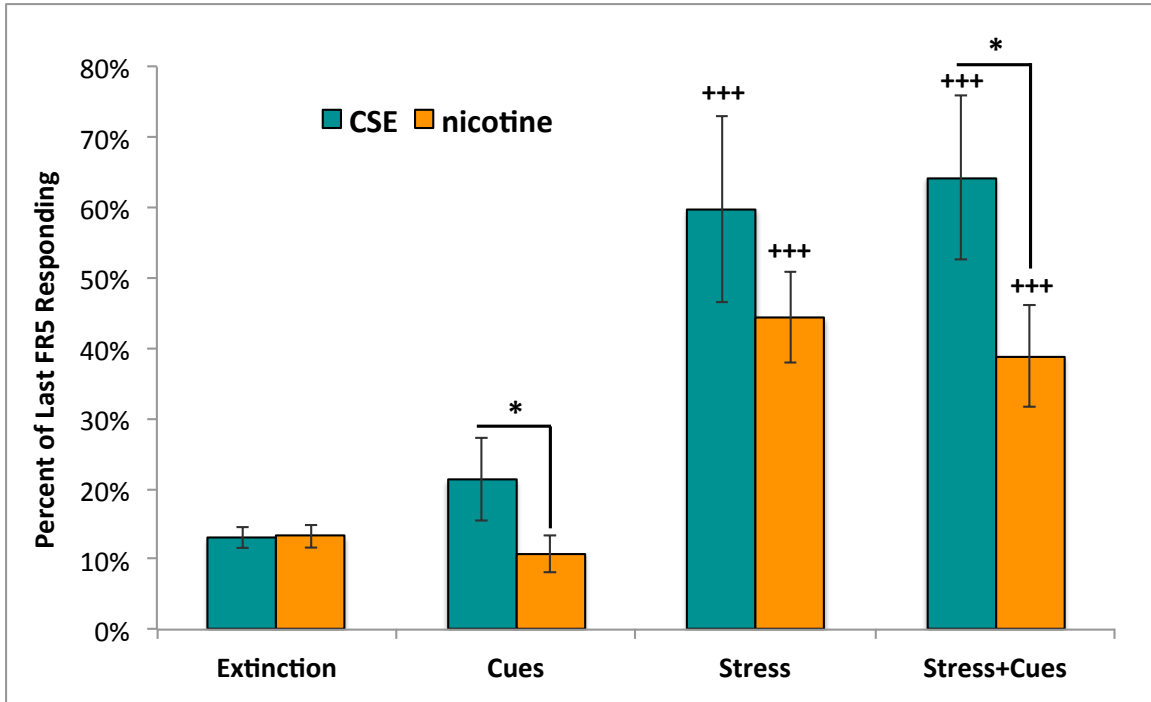**Figure 3.4. Drug, but not age, differences in stress- + cue-induced reinstatement.** The bars represent mean (±SEM) responding as a percentage of their last self-administration day for animals that had previously self-administered CSE (teal bars) or nicotine (orange bars). Since there was no age effect, adolescent and adult data were combined. +++ $p<0.0001$ vs. extinction; * $p<0.05$ vs nicotine. n=12-15 per group.
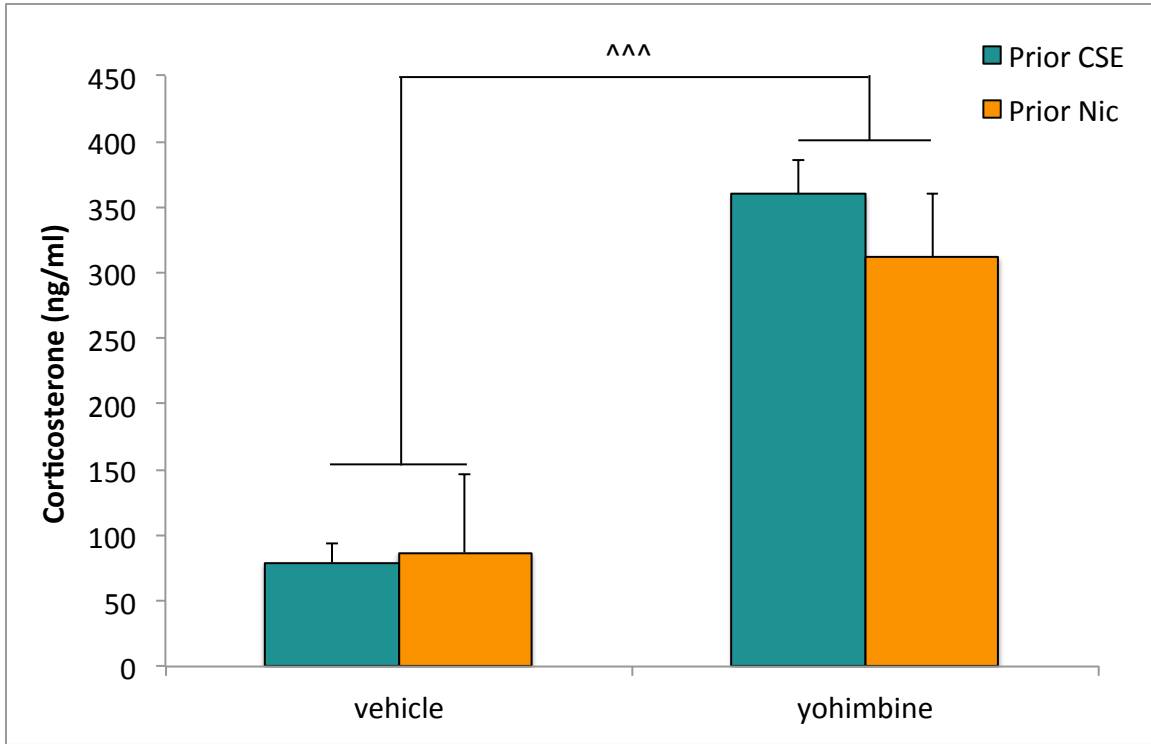
Figure 3.5. Age, but not drug, differences in yohimbine-induced HPA axis activation. The bars represent mean (±SEM) corticosterone levels after yohimbine or vehicle injections in adolescent and adult male rats (combined) that underwent stress-induced reinstatement of CSE- (teal) or nicotine-seeking (orange). ^^^ $p < 0.0001$ vs. vehicle. n=4-6 per group.

Chapter 4: Tobacco Smoke Constituents Do Not Alter α3β4 Nicotinic Receptor Mediation of Self-Administration in Adolescent Male Rats

Introduction

Of the 42.1 million Americans who smoke, 68% express a desire to quit (Center for Disease Control and Prevention (CDC) 2014). Only 7% of people are able to quit smoking without assistance (Hughes et al 2004). In order to help smokers quit, therapies such as nicotine replacement or pharmaceutical aids have been developed. Chantix (varenicline) is a pharmaceutical aid that has been shown to improve cessation rates, in which 23% of smokers were able to remain abstinent for over a year (Jorenby et al 2006). Although these aids begin to address the issues associated with smoking cessation, they still fail at helping the large number of remaining smokers who want to quit. To help those who continue to battle smoking addiction, novel pharmacotherapies are needed.

Recent work has focused on understanding how certain gene variants may influence nicotine dependence in order to identify novel targets for smoking cessation. Gene variants in CHRNA5-A3-B4, which encode for the α5, α3 and β4 nAChR subunits, have been shown to be closely associated with the risk for heavy smoking, inability to quit, and increased sensitivity to nicotine in humans (Berrettini et al 2008; Schlaepfer et al 2008; Weiss et al 2008; Caporaso et al 2009; Saccone et al 2009). In preclinical models, overexpression of the same gene cluster increases nicotine sensitivity and modifies its reinforcing effects in

mice (Gallego et al 2012). Furthermore, the α3β4 nAChRs have been shown to mediate nicotine reward in mice (Jackson et al 2013). Based on the overwhelming evidence suggesting α3β4 nAChRs are involved in nicotine dependence, a novel drug targeting these receptors, AT-1001, has been developed (Toll et al 2012; Wu et al 2014; Zaveri et al 2015; Cippitelli et al 2015).

AT-1001 has been shown to selectively bind to rat α3β4 nAChR and human α3β4α5 nAChR (Wu et al 2014). AT-1001 has partial agonist activity at the human α3β4 nAChR, which causes desensitization and evokes an inward current, resulting in an overall functional antagonism of α3β4 nAChRs (Zaveri et al 2015). Furthermore, AT-1001 has been shown to attenuate nicotine self-administration at doses in which it does not affect natural reward in rats (Toll et al 2012). These studies suggest that AT-1001 and compounds from this class target novel nAChRs and may provide a new pharmacotherapy for smoking cessation.

In order to fully understand how smoking cessation pharmacotherapies work, studies must focus on both adolescent and adult smokers. Although no pharmacotherapy for smoking cessation is currently approved for use in adolescents, clinical studies have shown that varenicline pharmacokinetics are similar in adolescents and adults, when adolescents are given half the dose to account for their smaller body weight (Faessel et al 2009; Faessel et al 2010). To my knowledge, no pre-clinical studies to date have investigated smoking cessation agents during adolescence. More in depth studies are needed to test whether varenicline and novel smoking cessation pharmacotherapies, such as

AT-1001, may aid adolescents in quitting. Furthermore, how tobacco smoke constituents may affect the efficacy of these pharmacotherapies is unknown in adolescents. In adults, it has been shown that AT-1001 only partially blocks CSE self-administration as compared to nicotine alone (Costello et al 2014). Based on this previous work and the fact that the non-nicotine constituents did not alter self-administration in adolescents in both of my previous studies, I hypothesized that AT-1001 would similarly attenuate CSE and nicotine self-administration in adolescents.

In order to investigate the impact of tobacco smoke constituents on novel smoking cessation pharmacotherapies during adolescence I have now compared AT-1001's attenuation of CSE or nicotine self-administration in adolescent male rats. As human adolescents are more likely than adults to initiate tobacco use and display a strong desire to quit (Karpinski et al 2010), it is vital to fully characterize novel smoking cessation pharmacotherapies like AT-1001 by expanding our studies to adolescence.

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 by bubbling smoke from commercial cigarettes (Camel unfiltered, RJ Reynolds) through sterile saline (Costello et al 2014; Gellner et al 2016b). Briefly, eight cigarettes were smoked through 35 ml of saline solution (35 ml puffs over 2 s, repeated every 30 s) and the final solution was adjusted to pH

7.2–7.4. The CSE solution was prepared fresh each day immediately before experimental testing in order to minimize differences resulting from differential stability of the constituents. All CSE doses were defined by the solution's nicotine content, which was analyzed by GC-MS (Finnigan Trace MS with Trace GC 2000 series, UCI Mass Spectrometry Facility). AT-1001, an $\alpha3\beta4$ selective nAChR ligand (Toll et al , 2012), was dissolved in 97% hydroxypropylcellulose (0.5% concentration in water), 2% DMSO, and 1% 0.1 M HCl.

Subjects

Adolescent male Sprague–Dawley rats were obtained from Charles River at postnatal (P) days 17 and remained with dam until weaning at P21. Animals were then group-housed throughout the experiment. All rats were maintained on a 12-h light/dark cycle (lights on at 07:00 am) with food and water available ad libitum. No more than one animal per litter per experimental group was used to avoid potential confounds. All experimental procedures were in compliance with NIH guidelines and were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

Rats were minimally food-restricted beginning two days prior to operant conditioning to promote exploration of the operant chamber and aid in acquisition of the operant task. Adolescent rats were fed 15–25 g of food to maintain normal growth during self-administration testing. Food was given 15 min after each experimental session, and any remaining chow was removed an hour before the following day test session. Food maintenance continued until the end of the

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experiment. Growth curves for adolescents followed normal trajectories (data not shown).

Behavioral Studies

Apparatus

Animals were tested in plexiglass operant chambers (Med Associates, St Albans, VT), equipped with two levers. Responses at the reinforced (R) lever resulted in illumination of a cue light over the lever and activation of an externally mounted syringe pump that infused drug. During the infusion (5.6 s yielding 100 μl of solution) and timeout period (20 s) the cue light remained illuminated and the house light was turned off. Responses on the non-reinforced (NR) lever were recorded but had no consequences.

Food Training

Adolescent rats, aged P25, were first trained to lever-press for food pellets (45 mg rodent purified diet; Bio-Serv, Frenchtown, NJ) under a fixed ratio 1 schedule with a 1 second timeout period (FR1TO1), followed by FR1TO10, FR2TO20, and completed with FR5TO20. Rats progressed to the next schedule when they earned at least 35 reinforcers in two 30-minute sessions per day.

Surgery

Following successful acquisition of food responding, rats were anesthetized with equithesin (0.0035 ml/g body weight) and implanted with indwelling jugular vein catheters (Belluzzi et al., 2005). During the 2-day recovery period, catheters were flushed daily with a heparinized saline solution to maintain

patency. The day before initiation of self-administration, and at intervals

thereafter, catheter patency was verified for rapid (5-10 s) anesthesia by infusing

propofol (5 mg/kg, i.v.). Patency was tested before self-administration, upon

stable self-administration, and before each dose of AT-1001 was administered.

Only animals showing rapid anesthesia at every stage were included in analyses.

AT-1001 Attenuation of Stable Responding

Adolescents, aged P37, were allowed to self-administer CSE or nicotine

(15 µg/kg/infusion nicotine content) or food pellets at the FR5TO20 schedule

during daily 1-hour sessions. Responding for nicotine, CSE, or food lasted for 10

days or until stable criteria were met (R within 20% of the mean over 3 days; R

>= 2 × NR responses; R >= 5). After reaching stable responding, AT-1001 (0,

0.75, 1.5, 3 mg/kg; s.c.; 10 min before session) was given in a within

counterbalanced design in which each dose was given in random order. Between

each dose, animals were allowed to self-administer nicotine, CSE, or food for two

days or until stable before undergoing another antagonist test.

Statistical Analyses

Adolescent self-administration at the FR5 schedule was analyzed using a

3-way ANOVA on R/NR x Day x Drug with repeated measures on R/NR and Day.

Significant main or interaction effects were further analyzed by a 1-way ANOVA

with Bonferroni-corrected paired (R/NR or Day) t-test post hoc comparisons. AT-

1001 dose response was analyzed using a 3-way ANOVA on Percent of Baseline

Responding x Dose x Drug with repeated measures on Dose. Significant main or

interaction effects were further analyzed by a 1-way ANOVA with Bonferroni-corrected paired (Dose) t-test post hoc comparisons.

Results

Initial self-administration

Adolescents were found to achieve stable self-administration of nicotine and CSE over an initial 10-day period (Figure 4.1). There was an overall main effects of Days [$F_{(9,144)}$=4.629, $p<0.0001$], but no overall or interaction effects of Drug were found (Figure 4.1). Post-hoc analysis showed that adolescents found the combination of CSE and nicotine reinforcing on all 10 days of the self-administration period in which they pressed the R lever significantly more than the NR lever ($p<0.0001$, Figure 4.1). Adolescents also increased their responding on the NR lever on Day 7 of the 10-day self-administration period ($p<0.05$, Figure 4.1).

AT-1001 attenuation of self-administration

Following stable self-administration, AT-1001 attenuation of nicotine or CSE self-administration was assessed in adolescents (Figure 4.2). An overall main effect of Dose [$F_{(3,48)}$=39.119, $p<0.0001$] was found, with no significant overall or interaction effects of Drug (Figure 4.2). Post-hoc analysis showed that AT-1001 dose dependently attenuated adolescent self-administration of both nicotine and CSE ($p<0.0001$).

To confirm that the effects of AT-1001 on self-administration were specific to drug reward, AT-1001 attenuation of food administration was conducted

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(Figure 4.3). Overall main effects of Dose [$F_{(3,12)}=3.534$, $p<0.048$] were found. However, post-hoc analysis did not show any significant differences between any test dose and the vehicle dose ($p=0.099$ for 3 mg/kg dose; Figure 4.3).

Discussion

Adolescence is a period of development in which teenagers who initiate tobacco use also display a strong desire to quit (Karpinski et al 2010). The smoking cessation pharmacotherapies that are currently available are only approved for use in adults. Pre-clinical studies are needed to test these pharmacotherapies in adolescents so that clinical approval for use in humans can be obtained. In order to fill the gap in pre-clinical studies I compared the effect of AT-1001, a novel smoking cessation pharmacotherapy, attenuation of CSE or nicotine self-administration in adolescent male rats.

As previously shown in Chapters 2 and 3, adolescents self-administer similar amounts of CSE and nicotine at an FR5 schedule. AT-1001 dose dependently attenuated both CSE and nicotine self-administration, indicating that the tobacco smoke constituents in CSE have no effect on adolescent self-administration behavior. Furthermore, the effect of AT-1001 is specific to attenuating drug reward, as it did not affect food reward at any dose tested.

Tobacco smoke constituent effects on attenuation of self-administration

As previously demonstrated (Gellner et al 2016a), tobacco smoke constituents had no effect on initial self-administration of CSE or nicotine in adolescents. The absence of tobacco smoke constituent effects during

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adolescence was further demonstrated by the fact that AT-1001 attenuated CSE

and nicotine self-administration to a similar degree. This contrasts with previous

findings in adults, in which AT-1001 attenuated nicotine self-administration to a

greater extent than CSE self-administration, suggesting that CSE and nicotine

reinforcement in adults may be mediated by different mechanisms (Costello et al

2014). My results suggest that the mechanisms that mediate CSE and nicotine

are similar in adolescents. Connections among reward-relevant regions continue

to develop during adolescence (Doremus-Fitzwater et al 2010). Furthermore,

differences in receptor binding and upregulation have been observed in

adolescents and adults (Doura et al 2008). These studies suggest that the

adolescent brain is distinctly different from the adult brain and may underlie the

differences observed between adolescent and adult attenuation of self-

administration. However, since the methods used in this experiment are different

than those used in Costello et al. (2014), adult experiments are currently

underway to fully explore the role of age in attenuation of CSE or nicotine self-

administration.

The role of α3β4 in mediating self-administration

AT-1001 is a novel smoking cessation pharmacotherapy that has been

shown to selectively bind to rat α3β4 nAChR and human α3β4α5 nAChR (Wu *et

al.*, 2014). Furthermore, AT-1001 has partial agonist activity at the human α3β4

nAChR (Zaveri et al 2015). The partial activation then causes desensitization,

evoking an inward current, resulting in an overall functional antagonism of α3β4

nAChRs (Zaveri et al 2015). AT-1001 does not significantly activate or

desensitize α4β2 nAChR at similar concentrations in which it partially activates α3β4 nAChR (Zaveri et al 2015). At 4-fold higher concentrations, AT-1001 does act as a partial agonist at human α4β2 nAChRs, with very low intrinsic activity suggesting that it does not significantly activate these receptors even at high doses (Zaveri et al 2015). A combination of these mechanisms may underlie the attenuation of nicotine (Toll et al 2012) and CSE (Costello et al 2014) self-administration by AT-1001, suggesting that AT-1001 and compounds from this class may have clinical potential for smoking cessation pharmacotherapy.

Conclusion

This study provides further experimental evidence implicating the importance of studying both the tobacco smoke constituents and age in animal models of tobacco dependence. Our laboratory has previously shown that AT-1001 partially attenuates CSE self-administration compared to nicotine self-administration in adults (Costello et al 2014). I now show that AT-1001 attenuates CSE and nicotine self-administration to a similar degree in adolescents. Further studies will be required to elucidate whether AT-1001, using the methods describe here, will differentially attenuate CSE and nicotine self-administration in adults. However, my current findings fill the gap in knowledge of how novel smoking cessation pharmacotherapies may prevent cigarette smoking in adolescents.
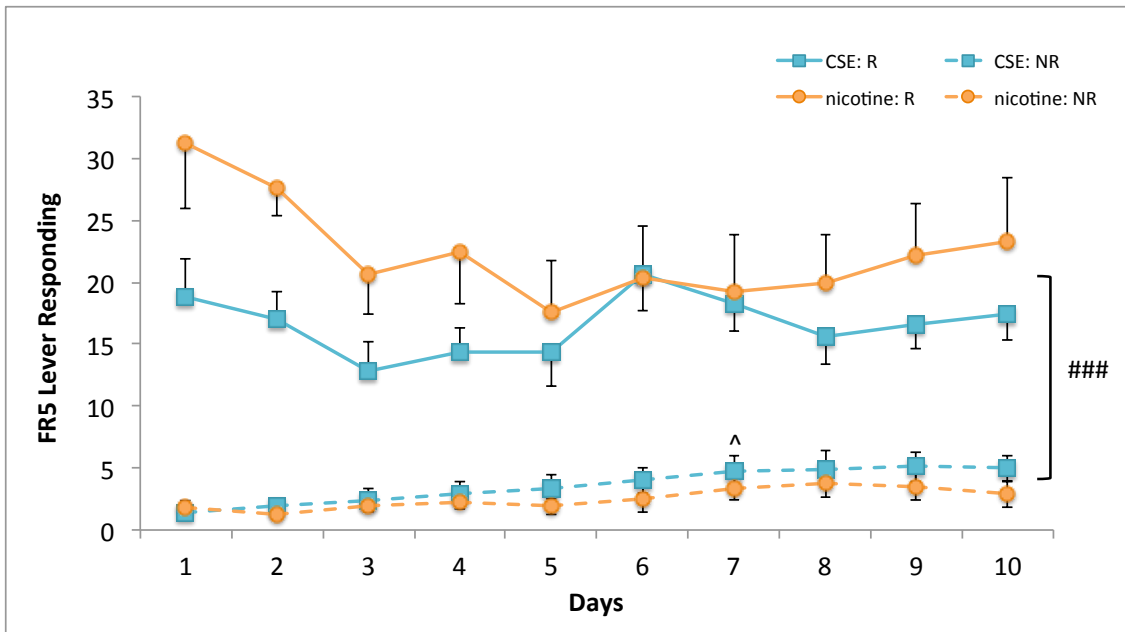
Figure 4.1 Adolescents self-administer similar amounts of CSE and nicotine. The lines represent mean (±SEM) responses leading to an infusion (R) of CSE (teal) and nicotine (orange) or responding in the non-reinforced lever (NR). *** p<0.0001 vs NR. ^ p<0.05 vs Day 1 NR. n=9 per group.
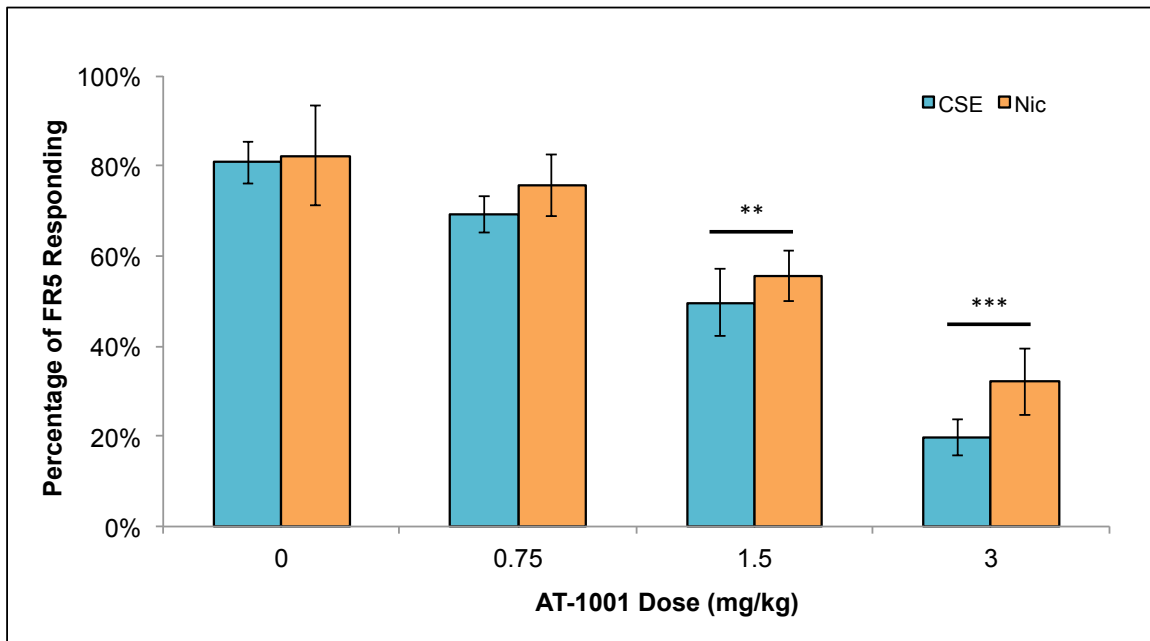
Figure 4.2. AT-1001 attenuates adolescent CSE and nicotine self-administration to a similar degree. The bars represent mean (±SEM) responding as a percentage of their last self-administration day for animals which previously self-administered CSE (teal bars) or nicotine (orange bars). *** $p < 0.0001$, ** $p < 0.01$, * $p < 0.05$ vs 0 dose of AT-1001, drugs combined. n=9 per group.

Figure 4.3. AT-1001 doesn't affect natural reward responding in adolescents.
The bars represent mean (±SEM) responding as a percentage of their last food-administration day. n=5 per group.

Chapter 5: Discussion

Cigarette smoking is the leading preventable cause of death and is responsible for one in five deaths annually, or 1,300 deaths every day (Alberg et al., 2014). Despite these well-known facts, every day approximately 3,200 peop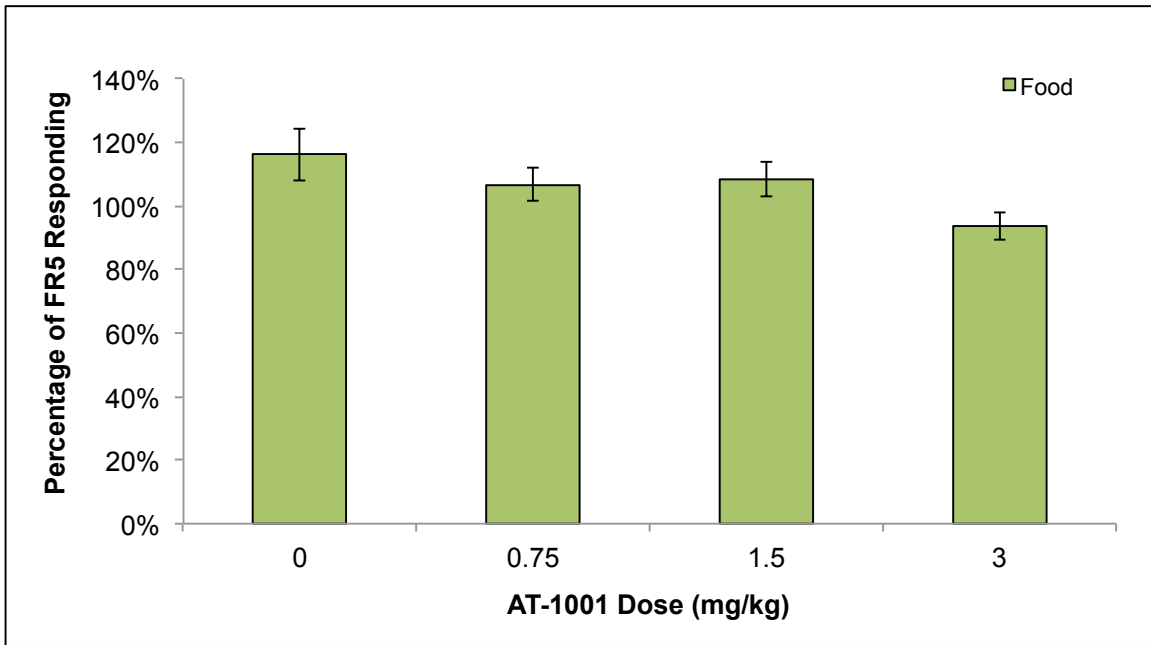le under the age of 18 try their first cigarette (Alberg et al., 2014). Although only 18% of the US population smokes and cigarette usage is on the decline, e-cigarette usage has been on the rise. The use of e-cigarettes exposes more adolescents to nicotine and increases their likelihood of becoming cigarette smokers (Dutra and Glantz, 2014). Nicotine exposure during adolescence produces unique behavioral responses, which may alter brain maturation and development (Spear, 2013, 2007; Yuan et al., 2015). However, traditional pre-clinical models of tobacco dependence ignore this important developmental period in which adolescents initiate tobacco use.

Over the last decade, there has been an increased understanding of the role of age in tobacco dependence. Adolescents are very different from adults in how they respond to nicotine. Adolescents readily acquire nicotine self-administration, take more nicotine, display enhanced sensitivity to the rewarding effects of nicotine, and do not find high doses of nicotine aversive compared to adults (Vastola et al 2002; Belluzzi et al 2004; Shram et al 2006; Kota et al 2007; Brielmaier et al 2008; Torres et al 2008). These studies illustrate the importance of including adolescence as a factor in studying tobacco dependence. However,

these studies still fail to include the more than 7,000 tobacco smoke constituents present in cigarettes.

To address this problem, scientists have begun to investigate the role of the non-nicotine constituents, either alone or in combination with nicotine. Monoamine oxidase inhibitors (MAOIs) are constituents found in tobacco smoke. To model MAO inhibition, tranylcypromine (TCP) is used and has been shown to enhance nicotine self-administration (Villégier et al 2007). TCP's enhancement of nicotine self-administration however was not age-dependent (Villégier et al 2007). A major constituent of tobacco smoke, acetaldehyde, was found to enhance the acquisition of early adolescent but not adult nicotine self-administration (Belluzzi et al 2005). These results begin to demonstrate the importance of studying both age and tobacco smoke constituents in animal models of tobacco dependence. Although a step in the right direction, studying one constituent at a time is laborious and neglects the potential interaction of the tobacco smoke constituents.

In order to get a more holistic picture of how the more than 7,000 constituents in tobacco smoke play a role in tobacco dependence, our lab created cigarette smoke extract (CSE), a solution containing the aqueous constituents in cigarette smoke. Adult rats have been shown to acquire CSE self-administration and do so at lower doses than nicotine alone (Costello et al., 2014). Furthermore, CSE has been shown to be more potent and to enhance stress-induced reinstatement compared to nicotine alone in adult rats (Costello et al., 2014). AT-1001, a novel smoking cessation aid, was only able to partially

attenuate adult CSE self-administration in which it fully attenuated nicotine self-administration (Costello et al., 2014). As CSE is a new tool for studying tobacco dependence, all experiments thus far used adult animals. The goal of my dissertation was to establish a proper model of tobacco dependence including both important factors involved in cigarette smoking, age and tobacco smoke constituents, in order to develop more efficacious smoking cessation aids.

Adolescents self-administer more low dose nicotine compared to adults

As most smokers initiate tobacco use during adolescence (Alberg et al 2014), my first question was whether the non-nicotine tobacco smoke constituents present in CSE would enhance acquisition of self-administration in adolescents. Based on previous data from our lab showing that acetaldehyde enhanced nicotine self-administration in early adolescence and that CSE enhanced acquisition of self-administration at low doses in adults, I hypothesized that CSE would enhance both adolescent and adult acquisition of self-administration compared to nicotine alone. I also hypothesized that adolescent acquisition of self-administration would be enhanced compared to adults.

In order to test my hypotheses, I developed a new method in which adolescent and adult acquisition could be compared. As adolescents have been shown to have high non-reinforced responding on nose pokes (Belluzzi et al., 2005), we chose to train animals to press a lever. To accommodate adolescents, animals were trained to an FR1 schedule before drug self-administration began. After 7 days on FR1, an FR2 schedule was used as a stepping stone to get to

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FR5, a schedule that can better assess the motivation to acquire drug administration. Using my newly developed acquisition paradigm, I found that adolescents were able to acquire self-administration of CSE. However, in contrast to what I hypothesized, the non-nicotine constituents did not enhance adolescent or adult acquisition of self-administration at any schedule or dose tested.

Consistent with previous data (Chen et al., 2007; Levin et al., 2007, 2003), I found that adolescents have higher nicotine intake compared to adults. This effect was at a low reinforcement schedule and was not enhanced by the presence of the non-nicotine constituents. Due to increased locomotion in adolescents (Belluzzi et al 2005), I investigated whether the increased nicotine intake in adolescents may be due to overall increases in activity. I found that adolescents had higher drug-induced non-reinforced responding compared to adults at both reinforcement schedules. When correcting for the differences in non-reinforced responding, I found that adolescents are more sensitive than adults to the reinforcing effects of low doses of nicotine at low reinforcement schedules only. As the reinforcement schedule became more challenging the age differences disappeared. Thus, the differences observed at FR1 were not robust.

Overall, my results suggest that adolescent and adult self-administration differs and that the non-nicotine constituents do not enhance acquisition of self-administration. My next question was whether these constituents alter other

phases of the tobacco dependence animal model such as the reinstatement phase, which models relapse to smoking.

Tobacco constituents enhance reinstatement of drug-seeking behavior in adults

Previous work in our lab has shown that CSE enhances stress-induced reinstatement of drug-seeking behavior in adult animals (Costello et al., 2014). Anker and Carroll (2010) showed that adolescents have higher stress-induced reinstatement of cocaine-seeking behavior compared to adults. Based on these studies, I hypothesized that adolescents would show enhanced stress-induced reinstatement of CSE- or nicotine-seeking compared to adults. Taking my acquisition of self-administration studies into account, I did not expect that tobacco smoke constituents would enhance reinstatement of nicotine-seeking behavior in adolescents. The first question I had to address was what dose of yohimbine should be used to induce similar amounts of stress in adolescents and adults as no dose response of yohimbine had been done at both ages.

Adolescence is a period of increased stress reactivity (Spear 2009), in which many factors such as type of stressor, length of exposure and time after exposure to stress differentially affect corticosterone release in adolescent and adult rats (Romeo et al 2006). To my knowledge, I was the first to examine corticosterone release after yohimbine exposure in both adolescents and adults. I found that both adolescents and adults show enhanced corticosterone levels after a 2.5 mg/kg dose of yohimbine. The levels of corticosterone at this dose of yohimbine were actually higher in adults compared to adolescents. As it was the

only dose of yohimbine that induced stress in both age groups, the 2.5 mg/kg dose was used to analyze stress-induced reinstatement of CSE- or nicotine-seeking in adolescents and adults.

In order to analyze stress-induced reinstatement, rats first stably responded for CSE or nicotine. Previous studies, including my acquisition study, have shown that adolescents take more nicotine compared to adults (Chen et al., 2007; Levin et al., 2007, 2003), a result that I replicated in my reinstatement study. In line with my first experiment (Chapter 2), I found that the non-nicotine constituents again did not enhance adolescent or adult self-administration of nicotine at a FR5 schedule of reinforcement. Also in line with results from Chapter 2, I found that adolescents displayed enhanced drug-induced non-specific activity compared to adults.

Previous studies indicated that adults initially extinguished slower after CSE self-administration compared to nicotine (Costello et al., 2014). I found that prior CSE self-administration did not enhance responding when adolescent and adult extinction was analyzed. In line with previous literature (Shram et al., 2008a), adults had an initial slower extinction compare to adolescents. Over the 5-day extinction period, adults decreased their responding to levels lower than that of adolescents. These results may suggest that adolescents actually learn that drug is no longer available quicker than adults. Furthermore, adolescents, due to their increased activity, maintain their responding over time so that when adults finally do learn that drug is no longer available, adolescents show higher responding toward the end of the extinction phase.

Upon extinguishing lever pressing behavior, animals were triggered to reinstate their drug-seeking behavior using known triggers such as stress and cues. I hypothesized that I would replicate previous work from our lab in which CSE enhanced stress-induced reinstatement in adults (Costello et al., 2014). I also hypothesized that I would not see this same enhancement in adolescents but that adolescent reinstatement would be enhanced compared to adults, based on work from Anker and Carroll (2010). Confirming my null hypotheses, I found that previous CSE self-administration enhanced stress+cue-induced reinstatement and that there were no effects of age on reinstatement of CSE- or nicotine-seeking behavior. I also found that CSE enhances responding for cues but not to a level that is significantly different from extinction, suggesting that the animals are not reinstating to cues but that CSE does enhance cue responding. These results may explain why the combination of stress and cues enhance reinstatement in animals, which previously self-administered CSE and not nicotine.

As previous work from our lab displayed enhanced stress-induced reinstatement of CSE-seeking behavior in adult animals (Costello et al., 2014), I questioned if this enhancement was due to enhanced HPA axis activation. To answer this question, I analyzed corticosterone release in animals that underwent a final yohimbine injection and re-exposure to the chamber to simulate reinstatement conditions. In order to account for the potential effect of increased lever pressing initially seen in adolescents, levers were removed. Corticosterone analysis revealed no effect of age or previous drug administration.

Yohimbine did enhance corticosterone levels suggesting that it was inducing activation of the HPA axis but that this was not the mechanism in which previous CSE self-administration enhanced stress-induced reinstatement of CSE-seeking behavior. My last question was whether α3β4 nAChRs were involved in CSE or nicotine self-administration in adolescents.

AT-1001 attenuates adolescent self-administration of CSE and nicotine

In order to answer my last question, I used a novel α3β4 functional antagonist, AT-1001. AT-1001 has previously been shown to attenuate both CSE and nicotine self-administration in adults (Costello et al., 2014; Toll et al., 2012). Furthermore, AT-1001 only partially attenuated CSE self-administration compared to nicotine self-administration (Costello et al., 2014). Based on this previous work and the fact that the non-nicotine constituents did not alter self-administration in adolescents in both of my previous studies, I hypothesized that AT-1001 would similarly attenuate CSE and nicotine self-administration in adolescents. First the animals stably responded for CSE or nicotine. I replicated my previous findings in which adolescent's self-administered similar amounts of CSE and nicotine over the 10-day self-administration period. They also pressed the reinforced lever significantly more than the non-reinforced lever, suggesting they found the drugs reinforcing. As before, the adolescents again increased their responding on the non-reinforced lever after administering drug.

When AT-1001 was given, I found that adolescents displayed a dose-dependent attenuation of CSE and nicotine self-administration. In line with my hypothesis, no effects of the non-nicotine constituents were found on AT-1001

attenuation of nicotine self-administration. In order to confirm that AT-1001 was in fact attenuating drug reward, food responding was also investigated. I found that AT-1001 did not attenuate food responding at the doses tested, suggesting that AT-1001 was specifically attenuating drug and not natural reinforcement. These results suggest that α3β4 nAChRs may be involved in CSE and nicotine reinforcement in adolescents.

Limitations and future directions

There are several limitations worth considering in this study. First and foremost is that the chemical composition of CSE is unknown. We know that CSE does not contain the non-aqueous constituents of cigarette smoke such as tar, which may have reinforcing properties and enhance the addiction resulting from smoking cigarettes. We do know that CSE contains some of the aqueous constituents from cigarette smoke, but we do not know how many of the 7,000 constituents we are extracting. However, the most important measure is the nicotine content within CSE. We have confirmed that nicotine is present via mass spectrometry and we analyzed the nicotine content of our CSE in order to ensure comparable amounts of nicotine in both the pure nicotine solution and the CSE solution. Further studies should focus on elucidating the exact composition of CSE.

Tobacco smoke constituent effects on acquisition of self-administration

To accommodate adolescent needs and promote discrimination, lever pressing and therefore food training was used to investigate the role of tobacco

smoke constituents in acquisition of self-administration. Traditionally, adolescent rats due to their high activity do not discriminate nose-poke holes in a timely manner (Belluzzi et al 2005). As adolescence is a relatively short time period, food training was used. As this was one of the first studies to use lever pressing in adolescents, they were only food trained to an FR1 schedule of reinforcement before being switched to drug. As animals were food maintained (adolescents) or restricted (adults), their motivation to press the lever for food was high, even when food was no longer present.

Over a seven-day period at the FR1 schedule, rats stabilize their intake of drug suggesting that they are no longer working for food, but are instead working for drug. Furthermore, rats continued to work for drug when the schedule was increased to FR5. However, the adolescents decreased their responding to adult levels. Future studies should explore whether the decrease in responding observed in adolescents is due to decreased motivation to work for drug by using a progressive ratio test.

Tobacco smoke constituent effects on relapse to smoking

Previous work in our lab indicated that previous CSE administration enhanced stress-induced reinstatement of CSE-seeking behavior in adults (Costello et al., 2014). My corticosterone results suggest this enhancement is not due to enhanced activation of the HPA axis. Furthermore, my reinstatement data indicated that CSE enhances cues in adolescents and adults. Further studies could investigate the role of cues in activation of the HPA axis. A lack of enhanced corticosterone release would indicate that HPA axis activation does

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not mediate CSE's enhancement of stress+cue-induced reinstatement. Another possible mechanism that could be tested is the role of the extrahypothalamic CRF system, which has also been heavily implicated in mediating stress responses (Koob 2010).

Tobacco smoke constituent effects on attenuation of self-administration

AT-1001 was use to elucidate the role of $\alpha3\beta4$ nAChRs in adolescent CSE self-administration. However, AT-1001 has also been shown to bind to $\alpha4\beta2$ nAChRs at concentrations 4 times higher than it binds to $\alpha3\beta4$ (Cipitelli et al., 2015). In addition, varenicline, an $\alpha4\beta2$ nAChR partial agonist, has been shown to also attenuate self-administration of CSE and nicotine in adults (Costello et al., 2014). Further studies would be required to test whether varenicline would also attenuate CSE and nicotine self-administration in adolescents. Furthermore, how AT-1001 attenuates adolescent and adult self-administration may be different. Due to a modified design from Costello et al. (2014) in which my animals only self-administered either CSE or nicotine, I cannot compare my adolescent data to his adult data. Therefore, experiments are currently underway to investigate whether AT-1001 differentially attenuates adolescent and adult self-administration of CSE or nicotine.

Conclusions

Data presented in my dissertation adds to the growing body of work implicating the importance of studying adolescence. Adolescence is an important developmental period in which smoking initiation occurs. My results show that

during this developmental period, adolescent rats self-administer more nicotine, regardless of the presence of tobacco smoke constituents. E-cigarettes may be a useful aid for smokers trying to quit because they lack the tobacco smoke constituents found in cigarettes. However, my findings suggest that e-cigarettes might increase the likelihood of cigarette initiation. Until this balance is established, it is difficult to determine the overall value of e-cigarettes and how they should be controlled. Furthermore, my findings stress the need for regulation of these products as animals relapsed after nicotine alone. Current pharmaceutical aids to help smokers quit are only 23% successful highlighting a need for novel targets. Furthermore, no currently available smoking cessation aids are approved for use in teenagers. My results suggest that AT-1001 may be a useful smoking cessation aid for use in adolescents. Overall, my findings provide novel insights on adolescent initiation and relapse and offer an exciting potential for the development of a new tobacco dependence animal model that could help create innovative therapeutics to curb the addiction faced by many.

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