Author

After noticing the spectrum of successes and failures that accompany smoking cessation, Emily Eshraghian developed a keen interest in the mechanisms of addiction, relapse, and withdrawal. Her curiosity led her to join Professor Leslie's lab, where she could explore the mechanistic differences of cigarette smoke cessation versus nicotine cessation. She particularly appreciates how her work has contributed to her growth as a student, researcher, and public health advocate; it has challenged her to think critically about scientific inquiries, while remaining receptive to the novelty required in research and medicine. After graduation, Emily will pursue her Master of Public Health in Epidemiology and aims to attend medical school.

Key Terms

- Antagonism
- AT-1001
- Attenuation
- CSE
- Extinction
- Nicotine
- Reinstatement

Attenuation of Drug-Primed Reinstatement by α3β4 nAChR Antagonism is Less Potent in Animals that Self-Administer CSE than Nicotine

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Abstract

Tobacco addiction is a chronic relapsing condition with negative impacts on public health. Nicotine is widely accepted to be the primary addictive component in tobacco; yet, current cessation therapies are ineffective. Many such therapies target α4β2 and α7 nicotinic acetylcholine receptors (nAChRs). However, other tobacco constituents also play a role. Studies have shown that α3β4 nAChRs function in nicotine dependence and provide a potential new target for smoking cessation. However, the role of α3β4 in drug-primed reinstatement (DPR) is still unclear. Here, AT-1001, an α3β4 antagonist, was used to examine its efficacy in attenuating DPR of a solution containing nicotine and the aqueous components of tobacco, cigarette smoke extract, (CSE)- or nicotine-seeking in rats. Animals self-administered CSE or nicotine for 10 days minimum before being extinguished from drug-taking behavior. Upon extinction, animals were treated with AT-1001, followed by a priming dose of CSE or nicotine prior to reinstatement testing. The study found that AT-1001 attenuated DPR of CSE- and nicotine-seeking in a dose-dependent manner, with lower efficacy in CSE animals. This demonstrates that α3β4 in part mediates DPR, which supports the evidence that the inclusion of tobacco constituents enhances craving. This highlights the importance of including tobacco constituents in preclinical models.

Faculty Mentor

This study shows that there is a difference in the mechanisms underlying craving following intake of nicotine alone and cigarette smoke extract. This is important because many teenagers and young adults are exposed to nicotine alone through e-cigarette use, rather than smoking cigarettes. The differences identified in the underlying pharmacological mechanisms may lead to new therapies for treatment of addiction caused by e-cigarette use. Emily's work shows the value of doing faculty-mentored undergraduate research. It teaches students to carefully design and conduct studies that can be of clinical impact.

Frances M. Leslie
School of Medicine

Objective

The goal of this study is to examine the effect of AT-1001, a selective antagonist of α3β4 nicotinic acetylcholine receptors (nAChRs), on attenuating drug-primed reinstatement of nicotine- or cigarette smoke extract (CSE)-seeking behavior in rats. We expect AT-1001 to be more robust at attenuating nicotine- than CSE-seeking behavior in rats.

Introduction

The use of tobacco has turned not only into an epidemic, but into one of the biggest public health threats facing the world, causing over 5 million deaths per year as the result of direct tobacco use (Tobacco, 2011). Although tobacco is present in products such as hookah and chewing tobacco, the tobacco found in cigarettes seems to be the most accessible form of tobacco induction among American youth. If smoking continues at the current rate, 5.6 million of today's Americans younger than 18 years of age are expected to die prematurely from a smoking-related illness (U.S. Department of Health and Human Services, 2014). Although aware of the harmful consequences associated with smoking, many smokers fail to eliminate cigarette usage. Of those who attempt to quit, 80% relapse within the first month of cessation, while only 3% remain abstinent after six months without support (Hughes et al., 1992). Even with the assistance of drug interventions and behavioral therapies, over 70% of smokers fail to remain abstinent for more than a year (George & O'Malley, 2004). These facts demonstrate the strong addictive potential of cigarette smoking, as well as the ineffectiveness of current smoking cessation therapies.

Tobacco addiction is a chronic relapsing condition distinguished by a tenacious craving to smoke regardless of its negative consequences or a user's desire to discontinue this habit (Bauzo & Bruijnzeel, 2012; Koob & Volkow, 2010; Lynch et al., 2010). In humans, the major hallmarks for relapse include craving of tobacco product(s) or a period of heighted stress (Doherty, Kinnunen, Militello, & Garvey, 1995; Koob & Volkow, 2010; Shiffman, Gorsline, & Gorodetzky, 2002; Swan, Ward, & Jack, 1996). The rate of relapse and abuse liability of tobacco are comparable to other drugs of abuse, such as opiates and stimulants (Anthony et al., 1994).

To better understand the neuro-mechanisms underlying craving and relapse, we turn to animal models. In laboratory animals, as in humans, the two most effective procedures for reinstatement of drug-seeking behavior after termina-tion of drug administration are (1) re-exposure to the drug, and (2) exposure to a brief period of stress (Stewart, 2000). Still, animal models of nicotine reinstatement do not readily predict the difficulty of abstinence maintenance in smokers, as they rely heavily on the presentation of drug-associated environmental cues.

Nicotine is widely accepted to be one of many addictive substances present in tobacco; it is the most studied psychoactive component of cigarette smoke, causing numerous preclinical studies to focus on the effects of nicotine alone (Costello et al., 2014; Foll & Goldberg, 2009). However, clinical studies have shown that the non-nicotinic constituents of cigarette smoke play a role in drug craving. In one clinical study, smokers were instructed to eliminate tobacco use for a short period of time (Rose, Behm, Westman, & Johnson, 2000); they were later distributed either de-nicotinized cigarettes, intravenous nicotine or saline infusions in order to rate the subject effects of the specific administered drug. Smokers reported that smoking the de-nicotinized cigarettes reduced their cravings and were significantly more rewarding than the non-smoking conditions (Rose et al., 2000). The intravenous infusions of nicotine, equal in dose to that of cigarettes, were reported to reduce cravings, but no significant satisfaction or feeling of reward was obtained (Rose et al., 2000). These results indicate that the non-nicotinic constituents in cigarette tobacco likely play a role in inducing symptoms of craving.

As noted, there is only mediocre validity in studies that show nicotine as the main/only addictive component of tobacco, due to nicotine replacement therapies (*i.e.* nicotine patches, gum, etc.) having low rates of continuous smoker cessation. In our lab, we use cigarette smoke extract (CSE), an aqueous solution containing nicotine and non-nicotine cigarette smoke constituents, as a tool to study tobacco dependence. Unlike nicotine alone, stress-induced reinstatement of CSE seeking was robust without the presence of cues (Costello et al., 2014). The extinction-reinstatement paradigm used to assess CSE-seeking behaviors is the first and only model to evaluate smoking in terms of relapse behavior in animal models. As demonstrated in prior findings, CSE sensitizes stress responses in animal models (Costello et al., 2014), designating that the non-nicotinic constituents of CSE are adding to the reinforcing value of nicotine. In preliminary data, we also show that animals that self-administer CSE show enhanced reinstatement of drug-seeking behavior after drug-priming, compared to animals that self-administered nicotine alone. This data exemplifies why CSE is a fitting model for relapse of smoking.

Current smoking cessation therapies, including Chantix (varenicline tartrate) and Zyban (buproprion hydrochloride), have been shown to target α4β2 and α7 receptors, which are distributed throughout the brain; due to this, such drugs can target large areas of the brain (where noted receptors are present) and induce side effects such as anxiety, depression, nausea, and suicidal thoughts (Ogbru & Stöppler, 2015). Recent studies have linked nicotine dependence to the α3β4 nicotinic acetylcholine receptor (nAChR) present in the medial habenula-interpeduncular pathway (IPN) (Frahm et al., 2011). The α3β4 nAChRs have a lower affinity for nicotine than the α4β2 receptors; they are likely less desensitized at the nicotine levels found in smokers than α4β2 receptors are (Frahm et al., 2011). Their retention of sensitivity to fluctuating nicotine levels in smokers indicates that α3β4 nAChRs could play an important role in tobacco addiction (Rose, 2007). In order to better examine this receptor, scientists have identified and characterized the first high affinity and selective α3β4 nAChR antagonist, AT-1001 (Toll et al., 2012). In a specific dose-response experiment, we found that AT-1001 significantly reduced responding to CSE in animals previously undergoing self-administration (Costello et al., 2014). Here, I propose to examine the potency of AT-1001 on attenuating drug-primed reinstatement of nicotine- or cigarette smoke extract (CSE)-seeking behavior in rats. Testing this will allow us to elucidate the role of α3β4 nAChRs in craving and relapse of tobacco use and evaluate AT-1001 as a novel therapeutic for smoking cessation. I hypothesize that AT-1001 will be more robust at attenuating nicotine- than CSE-seeking behavior due to the contributions that cigarette smoke constituents have on the addictive potential of nicotine.

Materials and Methods

Animals

This study uses Sprague Dawley rats, approximately 325-370 g in weight (Charles River Labs, Hollister, CA). Animals arrive at postnatal day (P)81 and are handled a day after arrival (for two minutes daily) for two days until initiation of the experiment. Adult male rats are housed in a humidity and temperature controlled vivarium with a 12-hour light cycle, with lights turned on at 7 a.m. daily. Animals are housed at two rats per cage. Water is readily available to rats at all times, excluding their time spent in the operant testing chambers. During the food training portion of the experiment, animals are restricted to 20–25 grams of food per day (distributed following their time in the operant chamber) in order to maintain 85% free body weight; once self-administration begins, animals are food restricted in order to maintain 95% free body weight. All experiments are approved by the UC Irvine Institutional Animal Use and Care Committee.

Surgery

Animals (P90–94) are anesthetized with a proper dose of equithesin (0.0035 mL/g body weight) and implanted with indwelling jugular vein catheters based on previously published methods (Belluzzi et. al, 2005). A 2-3 day recovery period is granted; during this period, animals are flushed with heparinized saline solution (1 mL of 1000 units/mL heparin into 30 mL bacteriostatic saline) daily until experiment initiation. Catheter patency is verified for rapid anesthesia via infusion of 0.1 mL of propofol (Abbot Laboratories, Chicago, IL) the day prior to day 1 of self-administration, as well as the day after the last day of self-administration.

Drugs

Nicotine Solution. Nicotine solution is prepared by dissolving nicotine hydrogen tartrate (Sigma, St. Louis, MO) in sterile saline; the solution is then adjusted to pH 7.2–7.4. All nicotine doses are calculated as free base amounts and diluted with 0.9% saline to ensure a correct dosage.

Cigarette Smoke Extract Solution. CSE is generated every morning by bubbling the smoke from commercial cigarettes (Camel unfiltered, R.J. Reynolds Co.) through sterile saline; this allows us to study the aqueous constituents in cigarette smoke (Figure 1). It is prepared by smoking unfiltered Camel cigarettes through a 1250 μL pipette tip and 50 mL conical tube filled with 35 mL of 0.9% saline solution. To
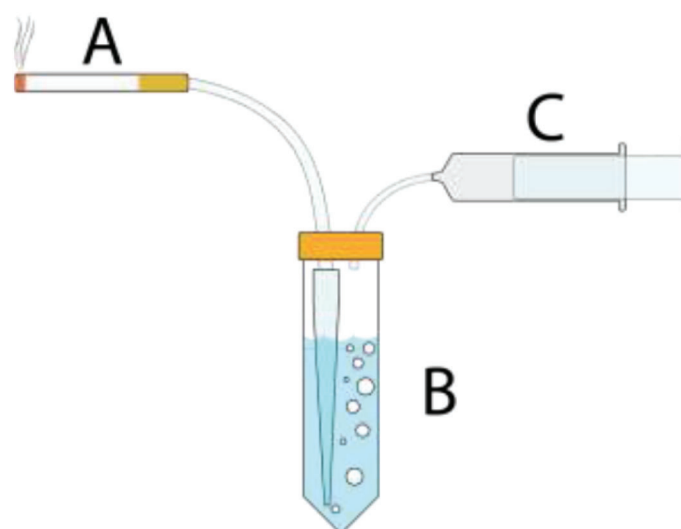
Figure 1

Preparation of cigarette smoke extract (CSE). Unfiltered cigarettes (A) are bubbled through a conical tube with 30mL sterile saline solution (B) by an experimenter-operated syringe (C).

mimic the reality of the human smoker, each cigarette is smoked for 2 seconds per 30 seconds, with a 35 mL "puff" (pull from the syringe) used to simulate smoker inhalation. Only 22mm of each cigarette is consumed, until a total of eight cigarettes has been smoked. The final solution is transferred into a new 50 mL conical tube and adjusted to a pH ranging from 7.2–7.4. CSE is analyzed for nicotine concentration to ensure that the content of nicotine available in the CSE solution is equivalent to levels present in the nicotine-only drug.

AT-1001 Drug Solution. The AT-1001 drug, provided by Dr. Nurulain Zaveri (Astrea Therapeutics, Mountain View, CA), is dissolved in 97% 0.5% hydroxypropylcellulose, 2% DMSO, and 1% 0.1M HCl.

Drug Treatment

Drug Self-Administration. Animals (P94–98) self-administer nicotine or CSE (15 µg/kg/infusion nicotine content) at a FR5TO20 schedule of reinforcement daily for one hour; this continues for a 10–15 days, until animals reach stable responding (reinforced responses (R) within 20% of the mean over the last 3 days; R ≥ 2 × non-reinforced (NR) responses; R ≥ 6). When stable responding levels are reached, extinction-reinstatement testing begins.

Extinction and Reinstatement. During extinction, animals are placed in the same operant testing chambers; the house light remains on, but animals are not connected to the infusion tubing, and the responses on the levels have no effects when pressed. Extinction periods occur for 5 days minimum at one hour daily, or until responding is reduced to 20% of baseline. After extinction, animals are triggered to reinstate drug-seeking behavior using five reinstatement conditions: cues, CSE or nicotine alone (0.15 mg/kg nicotine content, intraperitoneal (i.p.), immediately prior to the test) or CSE or nicotine in combination with cues in a counterbalanced design. Between reinstatement tests, animals are returned to extinction conditions for 2–3 days, until extinction criteria are met.

Drug Priming with AT-1001. Upon successful extinction, animals are treated with AT-1001. Four various doses of AT-1001 are administered to animals (0, 0.75, 1.5, 3.0 mg/kg; subcutaneous [s.c.]); ten minutes after injection and immediately prior to the reinstatement test, animals receive a priming dose of CSE or nicotine-only (0.15 mg/kg nicotine content; i.p). Animals undergo extinction in between each reinstatement test.

Data Analysis

To normalize data, both extinction and reinstatement data were analyzed as a percentage of baseline responding, calculated using the following equation: (Test day responding)/(Last day of FR5 responding) x 100. Mean responding was analyzed by a two-way ANOVA on drug × AT-1001 dosage with repeated measures on AT-1001 dosage. Significant main effects were analyzed further with appropriate post-hoc tests.

Results

The two-way ANOVA revealed significant main effects of AT-1001 dose on reinstatement ($F_{4,128} = 53.178$; $p = 0.000$) and drug ($F_{1,32} = 4.195$; $p = 0.024$) (Figure 2). Significant dose and drug interactions were also observed ($F_{8,128} = 2.913$; $p = 0.005$). All animals successfully reinstated at the 0 mg/kg of AT-1001 after drug-priming ($p = 0.000$ for all, vs. extinction; corrected paired t-test). AT-1001 dose-dependently attenuated drug-primed reinstatement in nicotine- and CSE-seeking animals; higher doses of AT-1001 were needed for CSE-seeking animals. The 0.75 mg/kg dose of AT-1001 attenuated reinstatement of nicotine-seeking ($p = 0.000$ vs. 0 dose; $p = 1.981$ vs. extinction; corrected paired t-test), but not CSE-seeking in CSE animals primed with CSE ($p = 3.731$ vs. 0 dose; $p = 0.028$ vs. extinction; corrected paired t-test) or nicotine ($p = 0.119$ vs. 0 dose; $p = 0.007$ vs. extinction; corrected paired t-test). At this dose, CSE animals, both nicotine-primed and CSE-primed, responded significantly higher than nicotine animals ($p = 0.013$, $p = 0.001$ respectively; bonferroni corrected unpaired t-test). AT-1001 attenuated reinstatement at the doses of 1.5 and 3 mg/kg in animals that had previ-
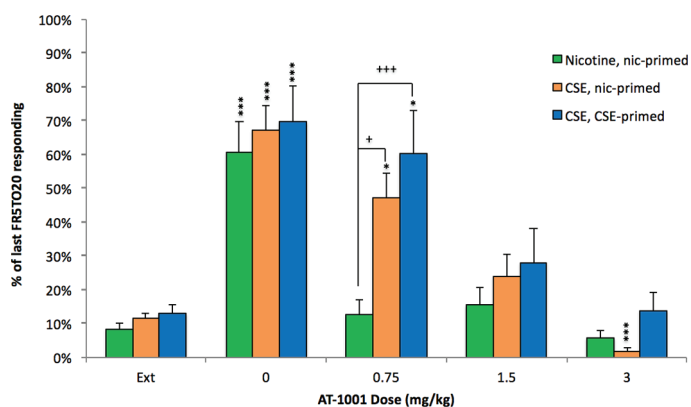

Figure 2

AT-1001 dose-dependently attenuates CSE- and nicotine-primed reinstatement with higher potency in animals that previously self-administered nicotine at the 0.75 mg/kg dose. *** = p < 0.001 vs extinction; * = p < 0.05 vs. extinction; +++ = p < 0.001; + = p < 0.05 vs. nicotine self-administration. n = 10–13 per group.

ously self-administered nicotine (p = 0.000 for both doses, vs. 0 dose; p = 1.127, p = 1.743 respectively, vs. extinction; corrected paired t-test) and animals that had previously self-administered CSE and were primed with CSE (p = 0.035, p = 0.000 respectively, vs. 0 dose; p = 1.19, p = 5.915 respectively vs. extinction, corrected paired t-test) and primed with nicotine (p = 0.000 for both doses, vs. 0 dose; p = 0.0749, p = 0.000 respectively, vs. extinction; corrected paired t-test) (Figure 2).

Discussion

These findings indicate that CSE self-administration enhances drug-primed reinstatement behavior by means of modified nAChR pharmacology. This is demonstrated by an enhanced nicotine-primed reinstatement responding and reduced attenuation of drug-primed reinstatement after α3β4 nAChR blockade with AT-1001.

This is the first set of experiments investigating the effects of cigarette smoke self-administration on drug- and cue-re-instatement tests. The reinstatement procedure is a widely used preclinical paradigm to study drug relapse. A similarity in factors that induce relapse in humans and reinstatement in animals, such as drug priming and the presentation of drug-associated cues, suggests acceptable causal validity for the reinstatement model. However, animal models of nicotine reinstatement do not readily predict the difficulty that smokers experience in maintaining abstinence. For instance, studies have demonstrated that animals that had previously self-administered nicotine required the presentation of drug-associated cues to reinstate after drug-priming (Caggiula et al., 2001; Sorge, Pierre, & Clarke, 2009). However, unlike with nicotine-alone, animals that had self-administered CSE reinstated without drug-asso-ciated cues; the presentation of cues further enhanced drug-seeking. This suggests that the inconsistency between the reinforcing potency of nicotine in preclinical models versus clinical studies may be due to the absence of other constituents found in cigarette smoke.

Nicotine replacement therapy (NRT) is one approach in the treatment of smoking cessation. Such therapies include over-the-counter treatments such as nicotine gum, patches, nasal spray, and electronic cigarettes. The goal of NRT is to provide nicotine to a smoker in the absence of tobacco, thereby relieving nicotine craving or withdrawal symptoms as the smoker breaks the behavior of cigarette smoking. However, clinical studies show that relapse rates in smokers who quit with NRT are similar to rates of those who quit without it (Sigaly et al., 2004). This study shows that a prim-

ing injection of nicotine alone also reinstated drug-seeking behavior in animals that previously self-administered CSE. This suggests that cigarette smoke constituents sensitize brain responses to nicotine, ultimately resulting in more intense craving. This may explain why nicotine replace-ment therapy is not an effective smoking cessation aid and perhaps refutes its effectiveness as a long-term smoking cessation aid.

Nicotinic receptors have been shown to have an import-ant role in mediating nicotine- and cue-induced reinstate-ment. For instance, the non-selective nAChR antagonist, mecamylamine, blocks nicotine self-administration and cue-induced reinstatement of nicotine-seeking as well as nicotine-primed reinstatement of conditioned place pref-erence (Biala, Staniak, & Budzynska, 2010; Costello et al., 2014; Toll et al., 2012; Liu et al., 2007). Furthermore, varenicline (a partial agonist of α4β2 nAChRs and agonist to α7 nAChRs) decreased, and in some cases increased, nicotine-primed reinstatement, while α7 blockade with methyllycaconitine, but not α4β2 blockade with DHβE, attenuated cue-induced reinstatement of nicotine-seeking in rats (Le Foll et al., 2012; Cippitelli et al., 2015; Liu et al., 2014). These studies highlight the specific role that different nAChR subtypes have on nicotine-primed and cue-induced reinstatement. Although much work has been done to inves-tigate the involvement of α4β2 and α7 nAChRs in drug- and cue-induced reinstatement, the role of α3β4 nAChRs has not been thoroughly investigated. These receptors are heav-ily expressed in the habenulo-interpenduncular (Hb-IPN) circuit; this tract is an important mediator of the aversive properties of nicotine, including the withdrawal syndrome following nicotine abstinence, which is closely linked to relapse (Gotti et al., 2009; Tuesta, Fowler, & Kenny, 2011). In agreement with published work, we demonstrated that AT-1001 blocks nicotine-primed reinstatement of nico-tine-seeking, emphasizing the importance of α3β4 nAChRs in drug-primed reinstatement to nicotine-seeking behavior (Cippitelli et al., 2015).

Since CSE animals showed enhanced responding to a priming dose of nicotine it seems likely that nAChRs are involved in the mechanism this enhancement displayed. Confirming this, AT-1001 dose-dependently attenuated drug-primed reinstatement in animals that self-administered CSE, but to a lesser extent than in animals that self-ad-ministered nicotine. The 0.75 mg/kg dose of AT-1001 attenuated nicotine-primed reinstatement in animals that previously self-administered nicotine, but that same dose did not attenuate reinstatement of CSE- or nicotine-primed reinstatement in animals that previously self-administered

CSE. Since the priming injection is given 10 minutes after AT-1001, this suggests a direct interaction of cigarette smoke constituents with nAChRs, perhaps sensitizing the receptor to its response to nicotine.

There are a few limitations in these experiments. One of the major challenges in studying smoking in animals is using a paradigm that best represents smoking in humans; our use of CSE is no exception to this boundary. CSE contains the water-soluble constituents of cigarette smoke; hence we are not accounting for the remaining ~60% of constituents in cigarette smoke (Schumacher, Green, Best, & Newell, 1977). Regardless, we have shown here that CSE is an improved model of studying relapse to smoking that will be valuable when assessing novel therapies for smoking cessation. As a second limitation, to investigate the role of α3β4 nAChRs in nicotine reinstatement, we paired drug and cue; this does not permit us to examine the role in drug-priming and cues independently. However, we cannot study them separately due to weak nicotine reinstatement subsequent to self-administration of 15 µg/kg/infusion nicotine dose. Third, differences in reinstatement after AT-1001 treatment are not explained with these studies. The difference may be from differences in nicotine or AT-1001 affinity to the α3β4 nAChR subsequent to chronic drug treatment. Previous work from our lab has shown no difference in nAChR's affinity after acute CSE or nicotine treatment, but it will be interesting to investigate whether differences arise after chronic treatment (Costello et al., 2014). Future studies may also investigate whether differences arise in α4β2 and α7 nAChR pharmacology as well.

In conclusion, we have demonstrated that the presence of aqueous cigarette smoke constituents in nicotine reinstatement studies contributes to the increased tendency for reinstatement, which occurs via a nicotinic receptor pathway involving α3β4 nAChRs. The results presented here suggest that nicotine is the primary constituent in CSE mediating drug-primed reinstatement. They also suggest that the inclusion of the aqueous constituents in CSE cause the enhancement of drug-primed reinstatement by sensitizing nAChRs to nicotine, thus leading to a decreased effect of α3β4 blockade after nicotine priming. These findings demonstrate the importance of including whole smoke constituents in preclinical models of tobacco dependence and relapse. They also suggest that α3β4 nAChR functional antagonism may be a suitable treatment approach to reduce nicotine and CSE craving during smoking cessation.

Acknowledgements

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