Mechanisms by which Nicotine Enhances Pavlovian Approach Behavior and Responding for a Conditioned Reinforcer

by

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Abstract

Tobacco dependence is among the leading causes of preventable deaths in North America. The main psychoactive ingredient contributing to the addictive properties of tobacco is nicotine. Nicotine use may be reinforced, in part, by an effect of nicotine to enhance the motivating properties of reward-associated stimuli. This effect can be measured in animals by first pairing a discrete stimulus with primary reinforcement in a Pavlovian conditioning procedure, making the cue a conditioned stimulus (CS). To assess the motivating properties of that CS, a second step is employed to assess the ability of the CS to serve as a conditioned reinforcer and support a novel, operant response. The number of responses performed for presentations of the CS as a conditioned reinforcer is enhanced by psychostimulant drugs, such as nicotine. In this thesis, I found that the administration of nicotine during Pavlovian conditioning enhances approach behavior towards the reward delivery receptacle when the CS indicates reward availability. This phenomenon occurred in two different Pavlovian conditioning procedures. In the second step, those animals that received nicotine injections during the Pavlovian conditioning phase displayed enhanced responding for the Pavlovian CS as a conditioned reinforcer under the influence of an acute nicotine injection. To further characterize this effect of nicotine on the reinforcing properties of CSs, I identified the specific nicotinic receptor subtypes involved in this effect, examined the longevity of responding for conditioned reinforcement, and assessed the ability of nicotine and the cues to reinvigorate this operant response after extinguishing such behavior. Furthermore, I found that drugs that act on

dopamine (DA) or serotonin (5-HT) receptors modify the effect of nicotine to enhance motivated responding for conditioned reinforcement. Finally, I assessed the impact of administering varenicline, bupropion, lorcaserin, and naltrexone on nicotine-enhanced responding for a conditioned reinforcer. Together, these data substantiate a role for nicotine in enhancing the motivating properties of CSs, and identify several pharmacological targets that influence this property of nicotine reinforcement.

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List of Abbreviations

5-HT:	serotonin
AMY:	amygdala
ANOVA:	Analysis of Variance
BOLD:	blood-oxygen level dependent
CS:	conditioned stimulus
CR:	conditioned reinforced lever
DA:	dopamine
DhβE:	dihydro-β-erythroidine hydrobromide
FDA:	Federal Drug Administration
fMRI:	functional magnetic resonance imaging
GABA:	gamma-amino-butyric acid
IP:	intraperitoneal
MLA:	methyllycaconitine
NAc:	nucleus accumbens
nAChR:	nicotinic acetylcholine receptor
NCR:	non-reinforced lever
NE:	noradrenaline/norepinephrine
NRT:	nicotine replacement therapy
PCS:	pre-conditioned stimulus

PFC: prefrontal cortex

RR: random ratio

SC: subcutaneous

US: unconditioned stimulus

VT: variable time

VTA: ventral tegmental area

VR: variable ratio

Chapter 1
General Introduction

It is estimated that approximately 19.3% of all adults in the United States smoke cigarettes on a regular basis (CDC, 2013). Tobacco use is associated with adverse health outcomes, such as coronary heart disease, chronic obstructive pulmonary disease (COPD), and cancer, making smoking one of the leading causes of preventable deaths in North America (CDC, 2013; George & O'Malley, 2004; Polosa & Benowitz, 2011). Nicotine is the primary psychoactive ingredient in tobacco smoke that reinforces smoking (Stolerman & Jarvis, 1995). Pharmacological interventions have shown some efficacy in abating tobacco use, but successful quit rates still remain low, hovering around the 20% range (George & O'Malley, 2004; Polosa & Benowitz, 2011). One reason that interventions may fail to curb smoking behaviors is an inability to mitigate the impact of certain factors that could perpetuate nicotine use. For example, some may continue to smoke to avoid dysphoric withdrawal symptoms (Cryan, Bruijnzeel, Skjei, & Markou, 2003; Warner & Shoaib, 2005) and some may smoke because of reported reductions in anxiety (Jonkman, Risbrough, Geyer, & Markou, 2008; Piasecki, Kenford, Smith, Fiore, & Baker, 1997). Some may persist in smoking primarily for its pleasurable (Epping-jordan, Watkins, Koob, & Markou, 1998; Kenny & Markou, 2006; Stolerman & Jarvis, 1995) or habit-inducing effects (Everitt et al., 2008; Stolerman & Jarvis, 1995). Considering these multiple possible psychological influences, a comprehensive understanding of the behavioral and pharmacological mechanisms that influence each of these factors contributing to smoking is needed. This information can be used to guide the development of new smoking intervention strategies that target these specific influences, or improve upon current treatments.

One characteristic feature of tobacco addiction is enhanced motivation to smoke in the presence of tobacco associated cues (Caggiula et al., 2001; Chaudhri et al., 2006; Chiamulera, 2005; Droungas, Ehrman, Childress, & O'Brien, 1995). These stimuli encourage ongoing nicotine consumption, or can trigger nicotine-seeking behavior during abstinence. The focus of this thesis is to further characterize some of the behavioral and pharmacological correlates of this influence over nicotine use. Towards this objective, this introduction first will provide a general overview of associative learning. The focus will be on reward-related associative learning, where stimuli associated with the availability of rewards become conditioned stimuli (CSs), and

can influence reward-directed approach behaviors as well as acquire reinforcing properties in their own right. Next, an explanation of how such associative learning can perpetuate drug use will be presented. Then, I will explain how animal studies of reinforcement learning can be used to identify behavioral and neuropharmacological correlates by which drugs may enhance the motivating properties of CSs. I will also provide an overview of the current pharmacological treatments used in nicotine-cessation therapy, and a discussion of how their neuropharmacological effects may influence the impact of nicotine on responding for conditioned reinforcement. This introduction will end with the specific aims of this thesis. These aims are to further elucidate how nicotine may influence learning about the motivating properties of CSs, the ability of these CSs to motivate and reinforce behavior, some pharmacological mechanisms of this aspect of nicotine reinforcement, and examine how pharmaceutical interventions may interact with nicotine to alter the impact of the conditioned reinforcing properties of CSs.

1 Reward-Related Learning

In general, learning can be defined as a process by which we can alter our skills based on prior experience, and it is revealed by changes in behavior (Eichenbaum, 2008). Learning can be described as either non-associative or associative. Non-associative learning does not require any specific associations between a stimulus and an outcome to be made in order to elicit a change in behavior. This type of learning includes reflex habituation, or a decrement in reflexive responses due to repeated stimulation by an effector stimulus. Presumably, this reduction in responding is due to diminished biological relevance of the effector stimulus. The classic example of habituation is a reduction in the gill-withdrawal reflex in response to repeated siphon stimulation in the sea slug *Aplysia* (Squire & Kandel, 1999). Non-associative learning also includes sensitization, or the amplification of a reflex response because of previous exposure to an arousing stimulus. For example, a reflexive startle response to a loud noise may be sensitized when it is preceded by a foot shock (Hitchock, Sananes, & Davis, 1989).

More recently, the term sensitization has been used more generally to describe enhancements in behavior after repeated exposure to a class of stimuli in the associative learning context, or learning that requires links to be formed between stimuli or a stimulus and response that are otherwise unrelated. Such terminology has been incorporated into theories of the development

of drug addiction (Berridge & Robinson, 1993), specifically the "Incentive Sensitization Theory of Addiction" (Robinson & Berridge, 1993). In this context, the term "sensitization" describes a phenomenon where individuals struggling with addiction become hypersensitive to the motivating properties of drugs and drug-associated stimuli, and consequently compulsively seek out drug reinforcement. Repeated exposure to drugs of abuse is argued to induce neuronal adaptations that can influence associative learning processes such that incentive motivation, or the drive to seek out reinforcement, is enhanced, or "sensitized." Of particular relevance to this thesis, the motivational significance of drug-associated CSs may become heightened in respect to other stimuli. Consequently, motivated responses (e.g., drug-seeking) in the presence of those stimuli appear to be sensitized. This thesis builds off the argument that the neuropharmacological effects of drug administration, specifically nicotine, may alter either reward related associative learning or incentive motivation elicited by reward-paired CSs, or affect both processes. These possible effects may influence the development of incentive sensitization in the presence of nicotine-associated stimuli.

2 Overview of Associative Learning

Associative learning is partitioned into two categories: classical, or Pavlovian, conditioning and response, or operant, conditioning. Pavlovian conditioning describes a type of associative learning where a relationship is formed between the presentation of an initially neutral stimulus and the availability of another stimulus with intrinsically rewarding or aversive properties that evokes a reflexive response in the organism. Presentations of these stimuli pairings are passive, in that their delivery does not depend on the behavior of the animal. Operant conditioning is similar to Pavlovian conditioning in that a relationship between a predictor stimulus and the delivery of a reflex-emitting stimulus is also formed, but in this case the organism must perform a specific behavior for the delivery of the reflex-emitting stimulus (Eichenbaum, 2008). Both types of conditioning procedures may alter subsequent patterns of behavior as animals acquire associations between stimuli and their outcomes. The presentation of certain behaviors may be increased or decreased, depending on whether they are associated with reinforcing or punishing outcomes. Reinforcing outcomes presumably induce pleasurable internal states, and increase the probability that a behavior emitted prior to the reinforcing event will be repeated. In contrast, punishing outcomes are likely associated with aversive internal states, and result in observed decrements in performing the behavior elicited prior to punishment (Skinner, 1938). This thesis

will focus on reinforcement learning in both Pavlovian conditioning, where discrete stimuli become associated with reinforcing outcomes, and operant conditioning reinforced by reward-associated stimuli.

3 Pavlovian Conditioning and Incentive Motivation

In Pavlovian conditioning, an initially neutral stimulus is repeatedly paired in close proximity to the delivery of an unconditioned stimulus (US) that has intrinsic rewarding or aversive properties (Pavlov, 1927). Encounters with the US evoke reflexive responses in the organism, that is, unconditioned responses (UR). Over time, an association between the CS and the US is made, and the initially neutral stimulus gains the ability to elicit the same responses in the organism as the US, thereby becoming a conditioned stimulus (CS).

In Pavlovian reward learning, the US may fulfill some metabolic need, such as water when in the thirst state, or food that provides needed calories in the hunger state (Hull, 1943). The US may also be intrinsically rewarding, as is often the case with drugs of abuse (Hull, 1943; Toates, 1986). Since these USs likely induce pleasurable states in the organism, they elicit approach responses to obtain them. Thus, if a neutral stimulus is repeatedly presented in close temporal proximity to the US, such that the stimulus predicts the US, presentations of the now CS will elicit approach responses that were formally reserved to presentations of the US (Skinner, 1938), and such behaviors are termed conditioned approach responses. During this behavioral transition, it is argued that a memory trace is developed representing the relationship between the CS, the delivery of the US, and representations of the anticipated hedonic value, based on prior experience with the US (Hull, 1943; Toates, 1986). Incentive motivation can therefore be influenced by the activated memory trace elicited by presentations of the CS (Berridge, 2001; Bolles, 1972; Robinson & Berridge, 1993).

However, enhancements in incentive motivation by CSs need not be specifically due to an activation of a specific memory trace regarding the anticipated incentive value of the US. Evidence suggests that the CSs themselves can also evoke general arousal states, and influence approach behaviors, regardless of current metabolic needs that may influence the incentive value of a reinforcer (Bindra, 1978). For example, a tone CS previously paired with food intake in the hunger state can later enhance food intake in sated animals (Holland & Petrovich, 2005). Likewise, a sucrose-paired tone CS can elicit more hedonic facial reactions in rats presently

consuming sucrose (Delamater, LoLordo, & Berridge, 1986). These studies suggest that animals are not necessarily evaluating the anticipated outcome of consuming the US based on their current metabolic or hedonic state, but instead are expressing a general enhancement in incentive motivation activated by the CS. Such effects may encourage reward-seeking if reinforcers are available in the sated state, creating adequate energy stores in case of future famine (Berthoud & Morrison, 2008). Thus, animals may not only emit more appetitive responses because of the expectation that US presentations will alleviate some deprivation state, but also because the CS itself can activate motivational states in its own right.

The acquired motivational significance of CSs is not just observed in the exhibition of conditioned approach responses. Incentive motivation elicited by the presentations of the CSs can affect behavior in at least three additional ways: (1) the CSs may invigorate ongoing reward-seeking behavior, (2) they may bias attention toward them, or (3) they may acquire reinforcing properties, and therefore become conditioned reinforcers. The acquired reinforcing properties of CSs is often measured by their ability to support operant learning (Everitt & Robbins, 2005; Saunders & Robinson, 2013), and is thought to reflect incentive motivation specifically evoked by presentations of the CS itself (Mackintosh, 1974). A major focus of this thesis is the influence of nicotine during Pavlovian conditioning on the acquired motivational significance of CSs, measured by the ability of the CS to reinforce a novel operant response.

4 The Influence of Pavlovian Conditioning and Conditioned Reinforcement in Perpetuating Drug Use

Pavlovian conditioning also extends to drug reward stimuli (Di Ciano & Everitt, 2004; Di Ciano, Robbins, & Everitt, 2008; Palmatier et al., 2007; Robinson & Berridge, 1993). As with natural reinforcers, drug-associated CSs can gain motivational significance, and support novel operant responses (Di Ciano & Everitt, 2004; Palmatier et al., 2007). The motivation-arousing effect of CSs likely influences several pathological behaviors involving reward-seeking and consumption, including overeating, drug abuse, and relapse to drug use after periods of abstinence (Caggiula et al., 2001; Chiamulera, 2005; Childress et al., 1999; Grimm, Hope, Wise, & Shaham, 2001; Robinson & Berridge, 1993; Tomie, Grimes, & Pohorecky, 2008). The sensitization of motivated behaviors exhibited in the presence of these stimuli is central to the Incentive Sensitization Theory of Drug Addiction (Robinson & Berridge, 1993).

Incentive Sensitization Theory argues that one way that drug use is sustained is by continued exposure to drug-associated CSs. The ability of these CSs to generate strong incentive motivation for drugs is thought to be reflected by subjective feelings of drug "craving" that powerfully motivate drug seeking behavior (Childress et al., 1999; Franklin et al., 2011; Kalivas & Volkow, 2005; Robinson & Berridge, 1993; Shaham, Shalev, Lu, De Wit, & Stewart, 2003; Weiss et al., 2000). This process may contribute to drug-seeking compulsions whenever salient drug CSs are present in the environment, and contribute to relapse in abstinent individuals (Everitt & Robbins, 2005; Robinson & Berridge, 1993). For example, showing videos of cocaine use to addicted individuals results in enhanced activity within brain regions associated with incentive motivation (Childress et al., 1999). This activity likely reflects increases in incentive motivation. Supporting evidence indicates that the degree of drug CS-associated activation within these same brain regions has also been linked to the probability of relapse, with heightened activity predicting shorter abstinence maintenance (Sinha & Li, 2007). Similar cue-elicited influences on drug seeking behavior occur in animals with a history of drug administration. For example, a CS previously paired with drug delivery can maintain rat drug-seeking behavior in the absence of the drug (Arroyo, Markou, Robbins, & Everitt, 1998; Caggiula et al., 2001; Ito, Dalley, Howes, Robbins, & Everitt, 2000), and these CSs reliably reactivate drug-seeking behavior after a period of extinguishing such responses (i.e., where drug-seeking responses are not followed by the delivery of the drug or CS; Kalivas & McFarland, 2003; McFarland & Kalivas, 2001; Shaham et al., 2003). Furthermore, parallel brain regions have been associated with both drug-cue induced craving states in humans and CS-induced relapse to drug seeking in rats (Kalivas & McFarland, 2003; Shaham et al., 2003), suggesting that similar neural processes are involved in the effect of CSs to elicit reward-seeking responses in both humans and rodent models of drug-taking behavior.

4.1 Evidence that Conditioned Stimuli Support Nicotine Administration

Like other abused substances, nicotine use is also influenced by motivationally-significant CSs, and tobacco addiction is argued to be influenced by Incentive Sensitization (Robinson & Berridge 1993; Berridge & Robinson, 1998). These incentive stimuli may be directly associated with the consumption of nicotine itself, such as the sight and smell of tobacco smoke, or they may often be present during nicotine use. As a legal substance, nicotine may be consumed in a

variety of different contexts. Thus, there are numerous opportunities to establish multiple CSs, making these cues particularly prevalent motivators of tobacco use.

4.1.1 Evidence in Humans

Exposure to nicotine CSs in laboratory settings arouses subjective feelings of "craving" (or ratings of desire to smoke) in abstaining smokers, and decreases the latency to smoke when cigarettes are again made available (Droungas et al., 1995; Franklin et al., 2011). Thus, these subjective cue-elicited cravings likely reflect increases in incentive motivation for tobacco. Stimuli that arouse such cravings can range from holding a cigarette, to the sight of cigarette packages, to cups of coffee, or even pictures of locations often associated with tobacco consumption (Baumann & Sayette, 2006; Droungas et al., 1995). Such smoking-associated stimuli may also influence motivation to smoke because they have acquired conditioned reinforcing properties through repeated association with nicotine consumption. Studies point to a role of tobacco-associated cues contributing to the subjective feelings of pleasure during tobacco consumption. Denicotinized cigarettes that look, feel, and taste like the smoker's preferred brand elicit pleasurable states, and are rated as "satisfying" upon consumption (Butschky, Bailey, Henningfield, & Pickworth, 1995; Rose, Behm, Westman, & Johnson, 2000). Some have shown that denicotinized cigarettes are also rated as more satisfying than the effect of a passive intravenous infusion of nicotine in the absence of these cigarette CSs (Rose et al., 2000), suggesting the importance of the conditioned reinforcing effect of these stimuli in maintaining tobacco use. Furthermore, some smoking behaviors, such as the level of carbon monoxide exhaled per cigarette puff, are insensitive to the removal of nicotine if the feel and sensations of smoking a tobacco cigarette remain unaltered (Butschky et al., 1995). In a recent report, nicotine was shown to enhance the reinforcing properties of a non-pharmacological stimulus, namely sections of preferred music played as a reinforcer for performing an effortful computer task (Perkins & Karelitz, 2013), suggesting that nicotine may also enhance the motivating properties of other reinforcers associated with its use. The effect of nicotine to enhance the motivating properties of other reinforcing stimuli can establish these objects as conditioned reinforcers of tobacco use. Together, such reports implicating nicotine-associated CSs in enhancing motivation to smoke and reinforcing tobacco consumption has contributed to the development of several theories specifically focusing on the contribution of tobacco-

associated CSs in nicotine addiction (Caggiula et al., 2001; Chaudhri et al., 2006; Chiamulera, 2005).

4.1.2 Evidence in Animals

Animals reliably self-administer nicotine with the aid of a CS associated with its delivery. At nicotine doses that are self-administered by animals, the introduction of a drug-paired CS synergistically increases response rates compared to levels maintained by nicotine or the cues alone (Caggiula et al., 2002). In animals trained to self-administer nicotine paired with a discrete CS, removal of that CS dramatically reduced response rates, and the reintroduction of the CS restored responding back to baseline levels of self-administration behavior (Caggiula et al., 2001). These nicotine-associated CSs also reliably reinstate self-administration behavior after periods of extinguishing such responding (Fletcher et al., 2012; LeSage, Burroughs, Dufek, Keyler, & Pentel, 2004; Liu et al., 2006). Furthermore, the ability of a nicotine-associated CS to reinstate self-administration behavior has, in some cases, been more effective than re-exposure to the drug (Caggiula et al., 2001; LeSage et al., 2004). For example, in a self-administration study where the CSs were removed and nicotine was replaced with saline, response rates on the lever that previously delivered nicotine were dramatically reduced. Reintroducing nicotine alone was ineffective in increasing self-administration behavior above these extinction levels, but the reintroduction of response-contingent presentations of the CS reactivated responding (Caggiula et al., 2001). Other reports have indicated that while a priming injection of nicotine itself did not affect extinguished responding, the presence of a salient stimulus, alone or in combination with nicotine, can reinstate self-administration behavior (LeSage et al., 2004). Together, these reports suggest that nicotine-associated CSs are at least as important as nicotine itself in maintaining drug-seeking behavior. The prominent role of CSs in nicotine reinforcement implies that research focused on the mechanisms underlying the conditioned reinforcing properties of these CSs is integral for understanding nicotine reinforcement in tobacco consumption.

4.2 Behavioral Procedures to Study the Interaction between Nicotine and the Motivating Properties of Conditioned Stimuli

The previous sections have highlighted the importance of nicotine-associated CSs in perpetuating smoking behavior in humans, and reinforcing nicotine self-administration in animals. However, stimuli associated with nicotine use in humans may extend beyond the

cigarette itself. For example, material presented in the previous sections suggests that stimuli associated with other reward objects (i.e., a bar, coffee cups, pleasurable music) can also become effective CSs motivating tobacco consumption (section 4.1.1), and nicotine may in fact enhance their motivating properties (Chaudhri et al., 2006; Perkins & Karelitz, 2013). A way to examine the interaction between nicotine and this general motivation-enhancing property in animal models is through repeatedly pairing an initially neutral stimulus with a natural reinforcer (US), such as water or food, using Pavlovian conditioning or related procedures, and examining the effects of nicotine administration on behavior.

4.2.1 Nicotine and Pavlovian Conditioned Approach

Pavlovian conditioning consists of passive presentations of an initially neutral stimulus paired with the delivery of a US, such as food or water. Presentations of the stimulus precede the US such that a relationship can be forged between the predictive value of the stimulus and the delivery of the US, and the stimulus then becomes a CS. Conditioned approach responses to the reward-delivery receptacle are monitored during presentations of the CS. Typically, the number of approach responses in the presence of the CS is compared to the number of approach responses in the absence of the CS (Olausson, Jentsch, & Taylor, 2003; Parkinson et al., 2002; Parkinson, Olmstead, Burns, Robbins, & Everitt, 1999; Wan & Peoples, 2008). The difference in responding during the CS periods compared to time periods when the CS is absent provides a measure of discriminated approach behavior, and this discrepancy in approach responding increases over time as animals learn the CS-US contingency. Nicotine has been shown to enhance discriminated approach behavior when it is administered either prior to the initiation of the entire Pavlovian conditioning procedure or following each Pavlovian conditioning session (Olausson et al., 2003). However, this effect was limited to the first three days of conditioning. In general, few published reports have examined the effect of nicotine on Pavlovian discriminated approach behavior, despite evidence supporting a substantial role for CSs in reinforcing nicotine seeking and consumption (Caggiula et al., 2001; Caggiula et al., 2002; Christian Chiamulera, 2005).

4.2.2 Pavlovian Autoshaping

Pavlovian autoshaping procedures differ from conditioning procedures that use a light and/or tone stimulus. In autoshaping, the CS is an object, typically a lever, which the animal can

interact with and manipulate. Traditionally, this procedure, which combines Pavlovian and operant conditioning, was used to "shape" animals to perform an operant response for the delivery of a reinforcer. After repeated CS-US pairings, animals approach the lever-CS, and often engage in appetitive responses directed at the lever-CS as if they are consuming the US (Jenkins & Moore, 1973; Silva, Timberlake, & Gont, 1998). Appetitive responding directed toward the CS is thought to represent a "generalized foraging response" for the primary reinforcer, and the CS is thought to serve as a substitute stimulus for the US (Jenkins & Moore, 1973). The likelihood of approach responses directed towards the CS can be altered by changes in location or alterations in the interstimulus interval length between CS and US presentations. CSs that are presented in close proximity to the delivery of the reward are more likely to be approached and engaged with as if they were the reinforcer itself. Also, as the time between the CS onset and US delivery is increased, approaches toward the CS itself are more likely (Silva et al., 1998). Thus, responding near or at the CS represents another form of conditioned responding that may show some variability during Pavlovian conditioning, but would not be captured using procedures where only approaches to the reward delivery receptacle are monitored.

More recently, this procedure has identified individual differences in conditioned approach responses. Animals are divided into groups based on their approach behavior toward the location of reward delivery in the presence of the predictive CS. Some animals develop approach behavior predominately directed towards the CS itself, and engage with the CS predictive of the reward as if it was a reinforcer (e.g., "gnawing" at a CS associated with food availability). These animals are categorized as "sign-trackers". Other animals develop approach behaviors focused toward the reward-delivery receptacle in the presence of the CS and are termed "goal-trackers." A third, "intermediate," group of animals display both types of conditioned responses with equal probability (Flagel, Watson, Robinson, & Akil, 2007; Robinson & Flagel, 2009). "Sign-trackers" have been shown to respond more for the CS as a conditioned reinforcer (Robinson & Flagel, 2009). Thus, this individual difference in conditioned approach behavior may reflect differences in the ability of the CS to elicit incentive motivation. Considering the evidence that nicotine enhances the motivating properties of CSs, nicotine may also affect this form of conditioned approach behavior by biasing conditioned responses toward the reward-predictive stimulus. A recent study indicated that nicotine injections prior to a Pavlovian conditioning procedure where the CS and US were presented in

locations that could be separately monitored resulted in nicotine enhancing approach responding toward the CS (Palmatier et al., 2013). However, there are no reported studies examining the possible influence of nicotine on approach towards a lever-CS during a Pavlovian autoshaping procedure.

4.2.3 Test of Responding for a Conditioned Reinforcer

Through conditioning, CSs acquire motivating properties in their own right, and can reinforce novel operant responses (Mackintosh, 1974). Performing such novel responses for a CS as a conditioned reinforcer is a strict measure of the degree of incentive motivation elicited by the CS because the operant response has never been paired with any other form of reinforcement (Everitt & Robbins, 2005; Robbins, 1975, 1978). Stimuli that have not been paired with reinforcement do not reliably reinforce the acquisition of a novel operant response, indicating that the association between this stimulus as a predictor of reward confers its motivational significance (Burton, Noble, & Fletcher, 2011; Taylor & Robbins, 1984; Beninger, Hanson, & Phillips, 1980). This procedure has been widely used to assess the impact of stimulant drugs as invigorators of incentive motivation (Beninger et al., 1980; Robbins, 1975, 1978; Taylor & Robbins, 1984). Nicotine, like other psychomotor stimulants, exhibits the property of enhancing responding for conditioned reinforcers (Olausson, Jentsch, & Taylor, 2004a), and prior, repeated nicotine exposure before testing can also enhance responding for conditioned reinforcers in the drug-free state, possibly reflecting incentive sensitization (Olausson, Jentsch, & Taylor, 2004b). Thus, nicotine may perpetuate smoking behavior not only by invigorating conditioned approach behaviors, but also by enhancing the motivating properties of reward-associated stimuli (Caggiula et al., 2001; Chiamulera, 2005).

4.2.4 Likely Neural Mechanisms Associated with Pavlovian Conditioning and Responding for Conditioned Reinforcement

In order to understand the possible neural mechanisms by which nicotine may enhance conditioned approach behaviors and responding for conditioned reinforcement, it is useful to examine the previous literature examining the neuropharmacological correlates of various forms of reward learning and drug reinforcement. Lesion and pharmacological inactivation studies in the absence of psychostimulant administration have helped characterize important mediating brain structures involved in forming the basic neural networks that support Pavlovian

conditioning and responding for conditioned reinforcement. Studies examining the impact of psychostimulants, such as cocaine and amphetamine, on responding for conditioned reinforcement have helped identify important neurotransmitter systems that confer motivational properties to CSs, and may be altered by drugs of abuse. Electrophysiological studies have helped characterize neural activation patterns within relevant brain regions that are associated with the development of memory traces between a CS and US during conditioning, and the effects of those CSs on motivated approach behaviors. Such studies provide important insight into the possible mechanisms by which nicotine interacts with reward-related learning during conditioning, and influences the motivating properties of CSs when they serve as conditioned reinforcers. In addition, prior research examining the administration of various centrally-active pharmaceuticals on drug and CS-reinforced behaviors provides candidate pharmacological targets to modify nicotine-induced enhancements in CS-evoked incentive motivation.

4.2.4.1 Neuroanatomical Correlates

Numerous pharmacological and lesion studies in rodents have associated activity within mesolimbic brain regions, specifically the nucleus accumbens (NAc), with the acquisition of conditioned approach behavior and invigorating responding for conditioned reinforcement. It has been argued that a major role of the NAc is to serve as a "limbic-motor interface" (Kelley, 2004) that receives from limbic brain regions associated with processing the motivational significance of reward objects, such as the amygdala (AMY) , prefrontal cortex (PFC), and ventral tegmental area (VTA), and then influences motivated approach behaviors by an influence over downstream motor output based on these motivational inputs (Kelley, Smith-Roe, & Holahan, 1997; Kelley, 2004; Parkinson, Olmstead, Burns, Robbins, & Everitt, 1999b; Taylor & Robbins, 1984). It is likely that the NAc is not necessary for forming CS-US relationships themselves because excitotoxic lesions to this region before Pavlovian conditioning do not prevent the acquisition of discriminated approach behavior (Hall, Parkinson, Connor, Dickinson, & Everitt, 2001), or the ability of the CS to serve as a reinforcer (Parkinson et al., 1999). Although some impairments in conditioned approach behavior following NAc lesions have been observed (Parkinson et al., 1999), the lesioned rats in those reports still preferentially approached the reward delivery receptacle when the CS is present. Thus, it may be possible that animals are still able to learn about the predictive value of the CS following damage to the NAc, but the activation of motivated approach responses in the presence of the CS may be diminished.

In contrast, structures that project to the NAc are likely more integral for storing information about the learned motivational significance of reward-associated stimuli (Cardinal, Parkinson, Hall, & Everitt, 2002). Previous reports have pointed to a prominent role for the AMY in storing such CS-US relationships. For example, lesions of the AMY not only impaired the acquisition of a conditioned approach response in the presence of a reward-associated CS, but also abolished preferential responding on a lever reinforced by that CS compared to a control lever with no associated consequences (Burns, Robbins, & Everitt, 1993). Furthermore, others have found that the AMY is necessary for storing specified information regarding the motivational significance of CSs based on prior experience. Rats trained to associate a CS with food availability when hungry showed enhancements in food consumption in the sated state when re-exposed to that same stimulus, an effect blocked by AMY lesions (Holland & Petrovich, 2005). In drug self-administration experiments, lesions to the AMY prior to extinction training in cocaine self-administering rats block the ability of the drug-associated CS to reinstate drug-seeking responses (Steensland, Simms, Holgate, Richards, & Bartlett, 2007), further implicating this structure in storing information about the incentive significance of CSs from prior experience.

The PFC may also be important for storing information regarding the motivational significance of reward-associated cues. Anatomically, the PFC innervates both the AMY and NAc, and these projections may be part of a mechanism where the PFC influences activity within these structures to modulate reward-directed behaviors (Cardinal et al., 2002; Kalivas & Volkow, 2005). A possible way that the PFC may influence reward-directed behaviors is by processing information regarding the CS and the anticipated incentive value of the associated reinforcer, for example, based on current needs and hedonic states, and flexibly altering incentive motivation based on sensory and visceral inputs (Cardinal et al., 2002). Several studies have examined PFC involvement in updating the anticipated incentive value of a reinforcer. In these studies, reinforcer value is experimentally manipulated by devaluing the reward by pairing it with gastric malaise, or pre-exposing the animal to the reinforcer prior to behavioral testing to induce reward satiation (e.g., pre-feeding before tests using a food reinforcer). In one such study, animals were trained to respond on one lever for one food reinforcer, and a different lever for a different food reinforcer. Excitotoxic lesions of the PFC blocked the effects of pre-feeding with a particular food reinforcer on reducing the response paired with the delivery of that reinforcer (Balleine & Dickinson, 1998). Lesions of the PFC, particularly the orbitofrontal PFC, also impair

performance of behaviors that require associations between specific discriminate stimuli, specific responses, and particular reinforcers. This can be observed using a Pavlovian-to-instrumental transfer (PIT) behavioral paradigm where, similar to conditioned reinforcement procedure, a CS gains the ability to motivate certain reward-seeking behaviors. In this procedure, first two discriminative CSs are each paired with the delivery of a different reinforcer. In a second step, animals are trained to perform one of two different operant responses for one of the two reinforcers initially paired with a discriminative CS in the first phase. The end result is that two different CS-response-reinforcer pairings are made. To test whether the appropriate pairings between a CS and a specific reinforcer have been established, one of the two learned responses is cued by the presentation of the discriminative CS that was paired with the particular reinforcer associated with the desired response. Lesions to the orbitofrontal PFC impaired the specific enhancement of the appropriate cued response (Ostlund & Balleine, 2007), implicating this structure in mediating the relationship between CSs and specific reward representations.

The previous studies describe behavioral procedures examining the role of the PFC in reward representations where food served as the reinforcer, but this brain region is also likely involved in processing the anticipated incentive value of drug-paired CSs, and may affect its conditioned reinforcing properties. In support, reversible inactivation of the dorsomedial PFC abolished the ability of a light/tone CS to reinstate drug-seeking behavior following extinction (McLaughlin & See, 2003), indicating that the PFC may play some role in modifying the conditioned reinforcing properties of drug-associated CSs. However, it is notable that lesions of the medial PFC do not block the ability of a CS paired with natural reinforcement to support the acquisition of a new response as a conditioned reinforcer (Burns et al., 1993). These discrepant effects, paired with the role for the PFC in processing reward outcome-expectancies, suggest that the PFC may not be directly involved in processing CS-reinforcer relationships. Instead, this structure may play a modulatory role in enhancing incentive motivation for a CS when it serves as a conditioned reinforcer, particularly for drug-associated stimuli. Therefore, the PFC may be one possible location where nicotine may act to invigorate responding for conditioned reinforcement.

The NAc, AMY, and PFC all send projections to the VTA, which in turn projects back to the NAc and then to motor output structures, forming a circuit to guide reward-directed behaviors (Everitt & Robbins, 2005). Changes in the environment that signal reinforcer availability (i.e., the presence of a CS), or alterations in incentive motivation (e.g., reward satiation), are

hypothesized to modify activity at one or more points along this circuit, and flexibly alter reward-directed approach behaviors. It is possible that the pharmacological effects of nicotine may influence neural processing within this circuit at one or multiple points to invigorate conditioned approach behaviors and/or responding for conditioned reinforcement.

4.2.4.2 Neurotransmitter Systems Associated with Incentive Motivation Elicited by the Presentation of Reward Associated CSs

Drugs of abuse act through diverse mechanisms to influence continued drug seeking and consumption. However, a common effect of administering virtually all drugs of abuse, including nicotine (Balfour, Wright, Benwell, & Birrell, 2000; Laviolette & van der Kooy, 2004; Markou, 2008; Ortells & Barrantes, 2011), is an associated enhancement of mesolimbic DA release (Di Chiara & Imperato, 1988b). While much research has focused on drug-induced changes in dopaminergic functioning, the serotonergic (5-HT; Fletcher, 1996; Higgins & Fletcher, 2003; Matteo, Giovanni, Mascio, & Esposito, 1999; Zaniewska, McCreary, Przegaliński, & Filip, 2007) and opiate systems (Di Chiara & Imperato, 1988a; Kelley et al., 2002; Peciña & Berridge, 2000; Spanagel, Herz, & Shippenberg, 1990; Spanagel & Weiss, 1999) have also been shown to modify drug reinforcement and motivated behaviors, likely by interacting with DA systems. Nicotine, through the activation of nicotinic acetylcholine receptors (nAChRs), also interacts with these neurotransmitter systems. Additionally, the excitatory neurotransmitter glutamate and primarily inhibitory neurotransmitter gamma-amino-butyric acid (GABA) are involved in incentive motivation and drug-seeking behavior, but their possible interactions with nicotine on Pavlovian conditioning and responding for conditioned reinforcement were not specifically examined in this thesis.

4.2.4.2.1 Dopamine

Changes in DA cell firing and release in the VTA-NAc mesolimbic pathway correlate with learning about the motivational significance of a CS (Berridge & Robinson, 1998; Clark, Collins, Sanford, & Phillips, 2013; Hollerman & Schultz, 1998; Lippa, Antelman, Fisher, & Canfield, 1973; Sutton & Beninger, 1999;). This effect may serve to promote neuroplastic changes that strengthen connections between the presentation of a CS and the generation of motivational states (Horvitz, Choi, Morvan, Eyny, & Balsam, 2007; Sutton & Beninger, 1999). Thus, DA release in the mesolimbic pathway could act as a "teaching signal" that strengthens the neural

networks mediating the relationship between the CS, the US, and the hedonic outcome of that US. As these relationships are established, subsequent presentations of the CS generate motivational states and elicit reward-seeking behaviors (Robinson & Berridge, 1993; Berridge & Robinson, 1998). Then, mesolimbic DA release in response to CS presentations may serve to strengthen networks involved in linking the CS to reinforcer representations and/or motivational states, and generating reward-seeking behaviors. This dual role of mesolimbic DA release in learning and motivation is reflected in electrophysiological studies of mesolimbic DA cell firing during Pavlovian conditioning. Initially, enhancements in DA cell firing are observed following the presentation of the reinforcer (US), but after CS-reinforcer (US) contingencies are established, the enhancements in DA activity follow presentations of the CS, rather than the reinforcer (Hollerman & Schultz, 1998; Horvitz et al., 2007; Robinson & Berridge, 1993; Salamone & Correa, 2012; Spanagel & Weiss, 1999). Additional supporting evidence for CS-evoked NAc DA release in incentive motivation following Pavlovian conditioning comes from fast-scan cyclic voltammetry studies, where synaptic DA levels can be monitored on a second by second basis as animals engage in reward seeking behaviors. In animals trained to associate a CS with the availability of a food reinforcer upon performing an operant response, presentations of the CS elicit elevations in synaptic NAc DA levels that are sustained as the animal approaches the lever and engages in the operant response. Once the operant behavior is performed, DA levels decrease until the next CS presentation, indicating that mesolimbic DA is also involved in incentive motivation processes evoked by reward-associated stimuli (Roitman, Stuber, Phillips, Wightman, & Carelli, 2004).

Similar to natural rewards, the presentation of cues associated with drugs of abuse elicit increases in mesolimbic DA release (Berridge & Robinson, 1998; Robinson & Berridge, 1993; Weiss et al., 2000). However, repeated exposure to drugs of abuse also sensitizes the mesolimbic DA response to administration of the drug (Paulson & Robinson, 1995), and it is hypothesized that repeated drug-seeking and taking likewise sensitizes mesolimbic DA response to CS presentations, conferring “incentive sensitization” and associated powerful urges to seek out the drug, even in the face of negative consequences (Everitt et al., 2008; Robinson & Berridge, 1993; Berridge & Robinson, 1998).

Reflecting the possibility of an incentive-sensitization effect in the presence of CSs following repeated drug use, repeated exposure to the psychostimulant cocaine, a dopamine reuptake

inhibitor, enhances Pavlovian discriminated approach behavior (Taylor & Jentsch, 2001). Similarly, repeated exposure to nicotine has been shown to facilitate discriminated approach responses (Olausson et al., 2003). Additional evidence for incentive-sensitization-like effects comes from studies that have examined the effects of psychostimulant administration on responding for conditioned reinforcement, a direct measure of incentive motivation. Numerous reports have indicated that dopamine receptor stimulation within the NAc, or drugs that enhance dopamine activity, increase responding for conditioned reinforcers (Beninger et al., 1980; Robbins, 1975, 1978; Taylor & Robbins, 1984). Furthermore, repeated exposure to cocaine also enhances the behavioral response to an intra-accumbens infusion of amphetamine in tests of responding for a conditioned reinforcer (Taylor & Horger, 1999), suggesting that behaviors activated by intra-accumbens infusions of DA receptor agonists become sensitized with a history of repeated psychostimulant drug exposure. Nicotine also exhibits this property of invigorating responding for conditioned reinforcers, and repeated nicotine exposure sensitizes responses to an intra-accumbens infusion of amphetamine (Olausson et al., 2004a; Olausson et al., 2004b).

In summary, psychostimulant drugs increase discriminated approach responses, and invigorate operant responding for conditioned reinforcement. These drugs also exhibit the common property of enhancing mesolimbic DA responses (Di Chiara & Imperato, 1988b), an effect that sensitizes with repeated exposure (Balfour, Benwell, Birrell, Kelly, & Al-Aloul, 1998; Paulson & Robinson, 1995). Considering that changes in mesolimbic DA responses are involved in forming CS-US associations (Clark et al., 2013; Hollerman & Schultz, 1998) and conferring incentive properties in these stimuli (Berridge & Robinson, 1998; Roitman et al., 2004), it is possible that repeated exposure to drugs of abuse may change DA firing patterns to alter the learned motivational significance during conditioning, and this effect may alter operant responding reinforced by the CS. However, this possible interaction has yet to be explored for psychostimulant drugs, including nicotine, and will be examined in Chapters 3 and 4 of this thesis.

4.2.4.2.2 Serotonin

5-HT receptors, particularly 5-HT_2 receptors, have demonstrated roles in influencing drug-seeking and relapse, presumably by facilitating (5-HT_{2A}) or blunting (5-HT_{2C}) NAc DA overflow in response to psychostimulant drug administration (Higgins & Fletcher, 2003; Navailles, De

Deurwaerdère, Porras, & Spampinato, 2004; Porras, Di Matteo, Fracasso, Lucas, & Spampinato, 1999). Consistent with evidence pointing to a DA suppressive effect following 5-HT_{2C} receptor stimulation, several studies have shown that agonists of this receptor subtype diminish drug-seeking behaviors. For example, studies of nicotine and cocaine self-administration have shown that systemic injections of 5-HT_{2C} receptor agonists decreased self-administration behavior on both fixed ratio and progressive ratio schedules of reinforcement. Similar effects are shown for cue-evoked motivated behaviors. Following extinction of responding on the drug-paired lever, 5-HT_{2C} agonists also blocked the reinstatement of self-administration behavior by a drug-paired CS, and these effects were reversed by the systemic administration of 5-HT_{2C} antagonists (Fletcher et al., 2012; Higgins et al., 2012; Neisewander & Acosta, 2004). Drugs that enhance synaptic serotonin levels, such as MDMA, reduce responding for conditioned reinforcement, an effect blocked by a 5-HT_{2C} antagonist (Fletcher, Korth, Robinson, & Baker, 2002). Together, these studies suggest 5-HT_{2C} receptors may decrease incentive motivation as assessed by responding for a conditioned reinforcer, and block the ability of nicotine to enhance such responding.

Since 5-HT_{2A} receptor stimulation is associated with facilitating mesolimbic DA release, antagonists of this receptor subtype are used to reduce drug-motivated behaviors. The systemic blockade of 5-HT_{2A} receptors reduces the effect of a nicotine or cocaine-paired CS to reinstate drug-seeking behavior following extinction. However, the effects of 5-HT_{2A} antagonism are not sufficient to reduce self-administration behavior when lever presses are reinforced by drug delivery (Fletcher et al., 2012; Fletcher, Grottick, & Higgins, 2002). Such evidence suggests that the blockade of these receptors may reduce some nicotine-motivated behaviors, but it remains to be determined whether 5-HT_{2A} receptor antagonism can effectively reduce nicotine-enhanced responding for conditioned reinforcement.

4.2.4.2.3 Opiates

Opiate receptors also play a modulatory role in enhancing or inhibiting mesolimbic DA release. Specifically, the peripheral administration of drugs that stimulate *mu*-opioid receptors enhance mesolimbic dopamine release, while *k*-opioid agonists reduce synaptic levels of dopamine (Di Chiara & Imperato, 1988a). Such opiate-induced enhancements in mesolimbic DA levels are associated with increased locomotor behavior (Di Chiara & Imperato, 1988a). This activating

property of opiate drugs may influence the ability of opiate-associated cues to become conditioned reinforcers that maintain self-administration behavior and reinstate drug-seeking after extinction (Stewart, de Wit, & Eikelboom, 1984). It is notable that mesolimbic DA release is not necessary for the reinforcing effects of opiate drugs themselves, since destruction of the mesolimbic DA terminals does not abolish heroin self-administration (Pettit, Ettenberg, Bloom, & Koob, 1984). However, mesolimbic DA receptor stimulation has been shown to mediate the reinstatement of self-administration behavior by opiate-associated contexts and discrete cues (Bossert, Poles, Wihbey, Koya, & Shaham, 2007). Therefore, the enhanced DA response associated with the stimulation of some opiate receptors, specifically the *mu*-opioid subtype, may specifically reflect incentive motivating properties of drug-associated stimuli (Berridge & Robinson, 1998; Berridge, 1996; Peciña & Berridge, 2000; Robinson & Berridge, 1993). In accord, *mu*-opioid receptor antagonists have been investigated in preclinical animal models of drug-relapse, and have shown some efficacy in interfering with the ability of a CS to reinstate drug-seeking behavior in self-administering animals self-administering amphetamines, alcohol, and even nicotine (Anggadiredja, Sakimura, Hiranita, & Yamamoto, 2004; Ciccocioppo, Martin-Fardon, & Weiss, 2002; Liu et al., 2009). In human smokers, *mu*-opioid receptor antagonists have been shown to reduce the effect of tobacco-paired cue presentations to elicit subjective cravings in human smokers (Hutchison et al., 1999). Thus, activation of opiate receptors, particularly the *mu*-opioid receptor subtype, may be involved in the motivating properties of CSs, and may interact with the effects of nicotine on responding for conditioned reinforcement.

4.2.4.2.4 Likely Neural Mechanisms Associated with the Effect of Nicotine on Conditioned Behaviors

The activation of DA, 5-HT, and opiate receptors all likely influence CS-elicited incentive motivation, and interact with the effect of nicotine to enhance the motivating properties of these stimuli. The pharmacological effects of nicotine are primarily mediated through activity at the nicotinic acetylcholine receptors (nAChRs). Eleven different nAChR subtypes have been identified (Picciotto, Caldarone, King, & Zachariou, 2000). These receptors have a pentameric structure and can be further classified as high-affinity heteromeric receptors comprised of both α and β subunits, or lower-affinity homomeric receptors containing solely α subunits (Besheer & Bevins, 2004; Fowler, Arends, & Kenney, 2006; Picciotto et al., 2000). Among the various nicotinic receptor subtypes, the activation of heteromeric α4β2 (Brunzell et al., 2006; Schilstrom,

Rawal, Mameli-Engvall, Nomikos, & Svensson, 2003; Threlfell et al., 2012) and homomeric α7 nicotinic receptors (Schilstrom et al., 2003) have been shown to alter activity of mesolimbic DA neurons. These receptor subtypes are localized in the VTA (α4β2 and α7), NAc (α4β2), and AMY (α4β2; Picciotto et al., 2000). Both subtypes are also located in the PFC (Gioanni et al., 1999) and hippocampus (Besheer & Bevins, 2004), and have roles in attention and memory processes (Besheer & Bevins, 2004; Grottick & Higgins, 2000). The distribution of these receptors suggests multiple possible sites where nicotine could influence Pavlovian conditioning and responding for conditioned reinforcement. However, most studies of nicotine reinforcement have focused on the activation of nAChRs within the VTA-NAc mesolimbic pathway (Corrigall & Coen, 1991, 1994; Corrigall et al., 1992; Laviolette & van der Kooy, 2003a, 2004).

Within the VTA-NAc pathway, the stimulation of homomeric α7 nAChRs is argued to be involved in nicotine reinforcement by increasing the burst firing rate of DA neurons (Schilstrom et al., 2003). This effect is hypothesized to be caused by enhancements in the firing rate of excitatory glutamatergic projections to the VTA DA cell bodies via a presynaptic mechanism (Besheer & Bevins, 2004; Markou, 2008). The α4β2 NAChR subtype has been shown to enhance the firing rate of mesolimbic DA neurons (Schilstrom et al., 2003) and to powerfully stimulate dopaminergic activity on the DA axons within the NAc, independently of the activation of DA neurons at the soma within the VTA (Threlfell et al., 2012). These receptors are also localized within the VTA-NAc reward pathway, specifically on VTA GABAergic inhibitory interneurons and the DA neurons themselves (Fowler et al., 2006; Markou, 2008; Picciotto et al., 2000). The ability of both receptors to modify DA release suggests these receptors dually contribute to nicotine reinforcement, supported by evidence from behavioral studies in animals (Corrigall, Coen, & Adamson, 1994; Grottick et al., 2000; Markou & Paterson, 2001; O'Conner et al., 2010).

The pharmacological blockade of both α7 receptors and α4β2 have been shown to reduce the number of nicotine infusions earned in self-administering animals (Corrigall, et al., 1994; Markou & Paterson, 2001). Furthermore, systemic administration of an antagonist at the α7 receptor subtype reduced responding on the lever paired with the delivery of the CS as a conditioned reinforcer (Löf, Olausson, Stomberg, Taylor, & Söderpalm, 2010), suggesting these receptors may also influence the reinforcing properties of CSs. However, the rats in the Löf et al. (2010) study were nicotine naïve. Evidence suggests that the α4β2 nAChRs, rather than α7

nAChRs, may mediate nicotine reinforcement with repeated exposure. Genetic manipulations that render the α4β2 nAChRs hypersensitive to nicotine activation enhance the expression of locomotor sensitization (Tapper et al., 2004), which may reflect some of the dopaminergic alterations that are involved in enhancing incentive motivation for the drug. Furthermore, others have found that the expression of locomotor sensitization after repeated nicotine injections was blocked by antagonists of the α4β2 receptor subtype, but not drugs that block α7 nAChR activity (Grottick et al., 2001). In studies that examined the effects of a history of nicotine exposure on the development of responding for CSs in mice, mice lacking the *β2 subunit did not exhibit such behavior (Brunzell et al., 2006). In a similar fashion, in experiments where repeated nicotine exposure enhanced responding for a visual stimulus, the effect of nicotine on this measure was blocked by α4β2, but not α7, nAChR antagonists (Liu, Palmatier, Caggiula, Donny, & Sved, 2007).

The α4β2 subtype, rather than the α7, is upregulated with repeated exposure to nicotine (Fowler et al., 2006; Govind, Vezina, & Green, 2009; Ortells & Barrantes, 2011), and this change reflects the sensitized mesolimbic DA response to repeated nicotine exposure (Balfour et al., 1998; Markou, 2008; Ortells & Barrantes, 2011; Threlfell et al., 2012). Since enhanced DA responses increase responding for conditioned reinforcers (Beninger et al., 1980; Kelley & Delfs, 1991; Robbins, 1975, 1978; Taylor & Robbins, 1984), it is possible that activity at α4β2, rather than α7 nAChRs influence the ability of repeated exposure to nicotine to induce enhancements in incentive motivation. Regardless, both receptors have been implicated in aspects of nicotine reinforcement, and an examination of both subtypes in nicotine-enhanced responding for a conditioned reinforcer is warranted.

5 Pharmaceutical Interventions for Smoking Cessation

5.1 Overview

Only a small number of smokers who attempt to quit are successful (George & O'Malley, 2004; Gonzales et al., 2013; Polosa & Benowitz, 2011). Numerous factors contribute to the perpetuation of smoking behaviors; including stress reduction, relief from boredom, anticipated energy from smoking, increased mood, and heightened desire to smoke following exposure to nicotine associated cues (Carmody, 2012; Ferguson & Shiffman, 2009; Piasecki et al., 1997). Despite these difficulties, smokers who use pharmaceutical interventions more than double their

quit rates (Gonzales et al., 2013). The property of nAChR stimulation to enhance mesolimbic DA release, and the major role for DA in various aspects of nicotine reinforcement, has led to the development of several pharmaceutical interventions that target either the nicotinic receptors themselves, or the DA system. To date, federal drug administration (FDA)-approved medications to aid with smoking abstinence include nicotine replacement therapy (NRT), the α4β2 nAChR partial agonist varenicline, and the DA and noradrenaline (NE) reuptake inhibitor bupropion (Polosa & Benowitz, 2011; Rigotti, 2013).

Unfortunately, these approved medications are associated with undesirable side effects, such as nausea, insomnia, heart arrhythmias, and increased risk of suicide (Polosa & Benowitz, 2011; Rigotti, 2013). These side effects, coupled with the limited efficacy of these agents in abating tobacco consumption, motivate research to better understand how these drugs may have their beneficial effects in order to improve upon them as well as generating research exploring novel treatment strategies. Proposed novel drug treatments target 5-HT and opiate receptors which, as described previously (Sections 4.2.4.2.2 and 4.2.4.2.3), may interact with the pharmacological and behavioral effects of nicotine. Examples of these proposed interventions include the 5-HT_{2C} receptor agonist lorcaserin and the *mu*-opioid antagonist naltrexone.

Considering that approved and proposed pharmaceutical interventions interact with mesolimbic DA responses, and presumably should affect incentive motivation, these drugs are likely to interact with nicotine's effects on responding for conditioned reinforcement. In this thesis, the effects of the approved drug interventions varenicline and bupropion, as well as the effects of proposed interventions lorcaserin and naltrexone, on nicotine-enhanced responding for conditioned reinforcement are examined.

5.2 Proposed Influence of Varenicline, Bupropion, Lorcaserin, or Naltrexone on Conditioned Behaviors Modified by Nicotine

The standard intervention to aid in smoking cessation is NRT, which works to reduce nicotine cravings by maintaining a steady blood serum level of nicotine (George & O'Malley, 2004). This is hypothesized to result in a reduction of withdrawal symptoms by maintaining nicotinic receptor tone during abstinence, and blocking receptor activation upon tobacco consumption (Polosa & Benowitz, 2011). However, this intervention strategy is controversial in that it requires individuals to cease all tobacco use immediately, due to potential toxicity issues from

heightened serum levels of nicotine. Individuals do not always comply with this request, and some are still capable of experiencing the acute effects of nAChR stimulation by continuing to smoke while on NRT. Furthermore, NRT may perpetuate dependence on nicotine itself, and the peripheral stimulatory effects of this substance may be damaging for individuals with heart disease (George & O'Malley, 2004). Thus, non-nicotine pharmaceutical compounds have been developed as an alternative, and their effects on conditioned reinforcement are examined in this thesis.

Varenicline (Chantix ®) acts as a partial agonist at the α4β2 receptor and a full agonist at the α7 receptor (Mihalak, Carroll, & Luetje, 2006). The effect of this partial stimulation of α4β2 receptors presumably mimics some of the motivational effects of nicotine during abstinence, but prevents nicotine from binding to these receptors. This presumably would block the reinforcing effects of the full agonist, nicotine (Coe et al., 2005; Levin et al., 2012; Rollema et al., 2007; Rollema et al., 2007). In support of this action, animal studies have shown that varenicline enhances responding for a reinforcing visual stimulus, and blunts the response-potentiating effect of nicotine on this behavior (Levin et al., 2012). It also blocks the ability of nicotine and a nicotine-associated CS to reinstate drug-seeking behavior in animals with a history of nicotine self-administration (O'Connor, Parker, Rollema, & Mead, 2010). Therefore, this drug may display a similar profile on responding for a conditioned reinforcer.

Another prescribed intervention to aid in smoking cessation is the atypical antidepressant bupropion (Zyban ®). The pharmaceutical effect of this drug is to enhance central dopaminergic and noradrenergic neurotransmission via blockade of the dopamine and norepinephrine transporters at the cell terminals. Increased synaptic availability of DA and NE may partially substitute for the influence of nicotine on these systems (George & O'Malley, 2004; Wiley, LaVecchia, Martin, & Damaj, 2002), and may enhance brain reward function to prevent negative withdrawal symptoms (Cryan, Bruijnzeel, Skjei, & Markou, 2003). Bupropion, like nicotine, also invigorates responding for a visual stimulus in rats (Palmatier et al., 2009), and may have similar effects on responding for conditioned reinforcers, therefore substituting for this aspect of nicotine reinforcement.

The weight-loss drug lorcaserin (Belviq®), has been presented as a possible intervention for cigarette smoking (Higgins, Sellers, & Fletcher, 2013). This drug is a 5-HT_{2C} agonist, and thus

has the property of attenuating mesolimbic DA responses. Rodent studies have shown that this drug reduces motivation for nicotine reinforcement, as well as cue and nicotine prime-induced reinstatement of nicotine seeking (Higgins et al., 2012; Levin et al., 2011). Similar reductions in responding for a conditioned reinforcer may occur following administration of this drug.

Last, the pharmaceutical naltrexone (Vivitrol ®), which is presently used to maintain alcohol abstinence (Garbutt et al., 2007), may provide relief from cravings during tobacco abstinence by reducing the effect of *mu*-opioid receptor stimulation to enhance cue-elicited DA release (Di Chiara & Imperato, 1988a; Spanagel & Weiss, 1999), thereby reducing nicotine-seeking behaviors. In support, there is some preclinical evidence that naltrexone reduces cue-evoked relapse to nicotine-seeking behavior in animals that self-administered nicotine paired with CS presentations (Liu et al., 2009). In summary, these four possible pharmaceutical alternatives to NRT have the potential to reduce, block, or substitute for nicotine-invigorated responding for reward-associated CSs.

6 Objectives and Hypotheses

The reviewed literature leads to several main conclusions. First, and most notably, nicotine can enhance at least some aspects of Pavlovian conditioned responding and incentive motivation. However, it remains to be determined whether nicotine administration during Pavlovian conditioning can affect Pavlovian approach behavior, and interacts with later effects on responding for the CS as a conditioned reinforcer. Second, the stimulation of dopamine and serotonin receptors can impact the reinforcing properties of reward-related stimuli, and likely affect the ability of nicotine to enhance the motivating properties of conditioned reinforcers. Last, nicotine-induced enhancements in the motivating properties for CSs likely contribute to continued nicotine use, and pharmaceutical interventions to reduce tobacco use and aid in abstinence may interact with this effect. Thus, it is possible that these interventions may alter nicotine-enhanced responding for a conditioned reinforcer. The experiments conducted in this thesis were designed to address these remaining questions regarding the behavioral effects of nicotine administration on Pavlovian conditioning and responding for conditioned reinforcement, as well as the influence of DA and 5-HT receptor activation and therapeutic drugs on this response. To address these questions, I tested the effects of nicotine exposure during Pavlovian conditioning on altering discriminated approach behavior to a goal object in the presence of the

CS compared to when the CS is absent, and the effect of nicotine administration on subsequent responding for conditioned reinforcement. Then, to determine if any effects on learning about the motivational significance of the CS during conditioning translate to differences in the ability of nicotine to enhance responding for a conditioned reinforcer, I compared the effects of nicotine exposure either early or late in the Pavlovian conditioning phase on responding for conditioned reinforcement. To identify the nicotinic receptor subtypes mediating nicotine-enhanced responding for conditioned reinforcement, I administered drugs that blocked nAChRs in general, and drugs that target the nAChR α4β2 or nAChR α7 receptor subtypes specifically. I also examined the possible influence of DA and 5-HT receptor activity on nicotine's behavioral effects by administering DA D_1 or D_2 antagonists, a 5-HT_{2A} antagonist, or a 5-HT_{2C} agonist prior to tests of responding for a conditioned reinforcer. Finally, I examined whether approved and proposed pharmaceutical interventions for smoking cessation were effective in modifying nicotine-induced enhancements in responding for conditioned reinforcement.

6.1 Aim 1: Examine the Possible Influence of Nicotine on Pavlovian Conditioning and Responding for a Conditioned Reinforcer and Identify nAChRs Mediating this Effect

The aim of the first set of experiments was to examine how nicotine administered just prior to Pavlovian conditioning sessions affected Pavlovian approach behavior and subsequent responding for CR in the presence of nicotine (Chapter 3). Prior research indicates that nicotine exposure either prior to the initiation of Pavlovian conditioning, or after each conditioning trial, enhances approach towards the reward-delivery receptacle, but only when the CS is present (Olausson et al., 2003). I expected to see a similar enhancement in discriminated approach behavior in animals exposed to nicotine prior to Pavlovian conditioning trials. Furthermore, I expected that the ability of nicotine to enhance responding for CR would be greater in rats previously exposed to nicotine during Pavlovian conditioning compared to the saline-exposed rats, based on evidence that some other behavioral responses to nicotine become sensitized with repeated exposure (Olausson, Engel, and Soderpalm 1999; Olausson et al., 2004b; Vezina, McGehee, and Green 2007).

I also examined the effects of pharmacological antagonism of nicotinic receptors with the non-selective nicotinic receptor antagonist mecamylamine, the competitive α7-containing nicotinic

antagonist methyllycaconitine (MLA), the competitive α4β2 antagonist Dihydro-β-erythroidine hydrobromide (DHβE) and on nicotine-enhanced responding for a conditioned reinforcer. My hypothesis was that blockade of nicotinic receptors would reduce the effect of nicotine to enhance responding for the conditioned reinforcer, based on prior research demonstrating roles for one or more of these receptors in nicotine-influenced behaviors (Brunzell et al., 2006; Grottick et al., 2000; Liu et al., 2007; Lof et al., 2010; Markou & Paterson, 2001; Olausson et al., 2004a).

6.2 Aim 2: Investigate the Potential Influence of Nicotine Exposure In Different Phases of Pavlovian Conditioning and Responding for a Conditioned Reinforcer

In the second set of experiments (Chapter 4), I examined the possibility that exposure to nicotine early or late in conditioning may differentially influence Pavlovian approach behavior and responding for the CS as a conditioned reinforcer. I hypothesized that nicotine would again enhance discriminated approach behavior, and that exposure to nicotine in the early conditioning trials would have a greater effect on discriminated approach compared to the later trials, based on previous reports that nicotine enhances reward learning in the early trials (Olausson et al., 2003). I also examined the influence of these different exposure regimens on the later ability of nicotine to enhance responding for a conditioned reinforcer, the extinction of such responding, and the effect of nicotine, the CS, or both stimuli to reactivate responding for the conditioned reinforcer.

The initial effect of nicotine to enhance Pavlovian discriminated approach was not apparent in this experiment, and I hypothesized that this may be because other types of conditioned approach behaviors were enhanced, such as approach toward the CS itself (Silva, Timberlake, & Gont, 1998; Flagel et al., 2007). Thus, I used a Pavlovian autoshaping procedure to examine the effect of nicotine on conditioned approach behaviors directed at the CS itself compared to the reward delivery receptacle during CS presentations. I also looked at the effect of different nicotine exposure schedules (early, late, or throughout conditioning) on approach behavior during autoshaping, as well as the ability of nicotine to enhance responding for the autoshaping CS as a conditioned reinforcer.

I hypothesized that nicotine would enhance approach behavior directed toward the autoshaping CS, particularly during the initial autoshaping trials. I also predicted that prior exposure to

nicotine would enhance responding for the autoshaping CS as a conditioned reinforcer, consistent with previous findings (Olausson et al., 2004b; Chapter 3).

6.3 Aim 3: Examination of the Effects of Dopamine or Serotonin 2A Receptor Antagonists, or the Stimulation of Serotonin 2c Receptors in Nicotine-Enhanced Responding for a Conditioned Reinforcer

To test the possibility that the administration of antagonists at D_1, D_2, or 5-HT_{2A} receptors, or the stimulation of 5-HT_{2C} receptors, would reduce the effect of nicotine to enhance responding for a conditioned reinforcer, animals were exposed to nicotine prior to each Pavlovian conditioning trial. This regimen reliably results in nicotine-enhanced responding for a conditioned reinforcer (Chapters 3 and 4). Then, I examined the effects of a pharmacological blockade of DA D_1 receptors with SCH 23390 or D_2 receptors with eticlopride on responding for the conditioned reinforcer, and the enhancement of such responding by nicotine. I also investigated the effects of the 5-HT_{2A} receptor antagonist M100907 or the 5-HT_{2C} receptor agonist Ro 60-0175 on responding for conditioned reinforcement and the ability of nicotine to enhance this response (Chapter 5).

Based on previous reports that these drugs reduce other types of nicotine and psychostimulant-modulated behaviors (Corrigall & Coen, 1991; Corrigall, Franklin, Coen, & Clarke, 1992; Fletcher et al., 2012; Higgins & Fletcher, 2003; Navailles, De Deurwaerdère, Porras, & Spampinato, 2004; Porras, Di Matteo, Fracasso, Lucas, & Spampinato, 1999), I hypothesized that administering SCH 23390, eticlopride, M100907, or Ro 60-0175 prior to testing would each reduce the effect of nicotine on enhancing responding for a conditioned reinforcer.

6.4 Aim 4: Investigation of the Possible Effects of Pharmaceutical Interventions for Smoking Cessation on the Ability of Nicotine to Enhance Responding for a Conditioned Reinforcer

To examine how pharmaceutical smoking-cessation aides may interact with this reinforcement-enhancing property of nicotine, the pharmaceutical interventions varenicline, bupropion, lorcaserin, or naltrexone were each administered in separate groups prior to a nicotine injection in the test of responding for a conditioned reinforcer.

I hypothesized that varenicline, lorcaserin and naltrexone would each reduce nicotine-enhanced responding for a conditioned reinforcer, based on previous studies indicating these drugs can block motivated behaviors influenced by nicotine administration (Higgins et al., 2012, 2013; Liu et al., 2009; O'Connor, Parker, Rollema, & Mead, 2010b). In contrast, I hypothesized that the stimulant properties of bupropion would enhance responding for a conditioned reinforcer. Prior research suggests that this drug can invigorate the reinforcing properties of some stimuli, and magnify the effect of nicotine on this behavior (Cryan, Bruijnzeel, Skjei, & Markou, 2003b; Palmatier et al., 2009; Shoaib, Sidhpura, & Shafait, 2003).

Chapter 2
General Methods

The experiments conducted in this thesis utilized two behavioral protocols to measure Pavlovian conditioned approach and the ability of a CS to reinforce a novel operant response as a conditioned reinforcer: the Conditioned Reinforcement procedure and Pavlovian autoshaping. First, I describe the general protocol and data recording for each procedure. Then, I identify the specific drugs and administration schedules for each experimental series. Finally, the general statistical approach is described.

1 Subjects

Male Long-Evans rats (Charles River, Quebec, Canada) weighing 225-250g upon arrival were singly housed in a temperature (~22°C) and humidity-controlled (~50-60%) vivarium on a 12 hour light/dark cycle (lights on 0700 h-off 1900 h). Food was available at all times, but water access was restricted as described below. All procedures were approved by the Centre for Addiction and Mental Health Animal Care Committee and adhered to Canadian Tricouncil guidelines for the humane treatment of experimental animals.

2 Water Restriction

All behavioral testing was conducted in the water-deprived state, unless otherwise noted. Water restriction was initiated ~23 h prior to the next behavioral session. Access to water was given approximately 20 min after each behavioral procedure for 1 h, and 24 h access was given during any intervening days.

3 Equipment

Training and testing occurred in sound-attenuated operant conditioning chambers (Med Associates, St. Albans, VT, USA) configured with two retractable levers located 6.5 cm from either side of a recessed water receptacle positioned 3 cm from the floor of the chamber. Water was delivered by a solenoid operated water dispenser. A red stimulus light was located above each retractable lever and a Sonalert sound generator and white houselight were located at the rear of the chamber opposite the water magazine, with the exception of Pavlovian autoshaping.

During autoshaping, the houselight was changed to red and the left lever was illuminated by an LED light.

4 Behavioral Procedures

The specifics of these general procedures are elaborated in each chapter. Briefly, variations of the following behavioral procedures were used for each of the studies in this thesis.

4.1 Responding for a Conditioned Reinforcer

This behavioral method was applied in all experiments, with the exception of Chapter 4, Experiment 2. The procedure consists of two phases: a Pavlovian phase where animals learn to associate a light/tone conditioned stimulus (CS) with the delivery of water reinforcement and an operant phase where animals must acquire a novel lever-pressing response for subsequent presentations of the light/tone CS.

4.1.1 Pavlovian Conditioning

The day prior to the initiation of Pavlovian conditioning sessions, animals were restricted to 1 h of free water access and remained water-restricted throughout conditioning and testing procedures. Each 30 min Pavlovian session consisted of 30 pairings of a 5 s CS followed immediately by the presentation of 0.05 mL of tap water (US) on a random time (RT) 60 s schedule of reinforcement. The CS consisted of a 5 s illumination of the two red stimulus lights with the houselights turned off and a 2.9 kHz, 85 dB tone stimulus presented during the last 0.5 s of the light presentation.

4.1.2 Responding for a Conditioned Reinforcer

During tests of responding for a conditioned reinforcer, 2 levers were inserted into the chambers. Responding on one lever resulted in presentation of the CS, in the absence of the water reward, on a variable ratio (VR) 2 schedule of reinforcement. Since this CS now functioned as a conditioned reinforcer, this lever was designated the CR lever. Responses on the other lever, designated the NCR lever, had no programmed consequences. CR and NCR lever responses were recorded.

4.2 Pavlovian Autoshaping Procedure

4.2.1 Overview

This procedure consisted of two phases and was conducted solely in Chapter 4, Experiment 2. First, animals learned to approach and engage with an illuminated lever-CS that was inserted into the chambers prior to a water reinforcer. In the second phase, animals acquired a nosepoking operant response for presentations of the lever-CS previously associated with water.

4.2.2 Pavlovian Autoshaping

Testing took place in the same operant chambers used in the Conditioned Reinforcement procedure. However, a red houselight was used and remained switched on throughout the session. During each session, 25 CS-US pairings were delivered on a RT 90 s schedule of reinforcement. The CS consisted of the insertion of the left retractable lever into the chamber, backlight illuminated by a flush-mounted 0.635 cm high output LED light. After 8 s, the lever was retracted and 0.05 mL of tap water (the US) was delivered to the central water receptacle. Sessions took place at the same time each day and lasted approximately 45 min each. In all sessions, the number of contacts with the CS (lever) and the number of nose-poke responses in the water receptacle during CS presentations were recorded. The number of responses in the water receptacle in the absence of the CS was recorded separately.

4.2.3 Responding for an Autoshaping CS as a Conditioned Reinforcer

After Pavlovian autoshaping sessions, all animals underwent 40 m tests of responding for conditioned reinforcement. For this procedure, the illuminated retractable lever was moved to the center panel of the conditioning chamber, in place of the water receptacle. Two nosepoke ports were placed equidistant apart on either side of the lever. Nosepokes into the reinforced (CR) port resulted in a 2 s presentation of the illuminated lever. Nosepokes into the other port (NCR port) were recorded, but had no programmed consequences. Responses on the illuminated lever during conditioned reinforcer presentations were also recorded.

5 Drugs

All doses are expressed as the base amount of drug. [-]-nicotine bitartrate (Sigma, St. Louis, MO) was dissolved in sterile 0.9% saline and titrated to a pH of ~7.2 and injected (0.4 mg/kg,

subcutaneous-SC) 5 m prior to all behavioral tests. The non-competitive nicotinic receptor antagonist mecamylamine (Tocris Bioscience, Ellisville, MO), the primarily α7 nicotinic receptor antagonist MLA (Tocris Bioscience, Ellisville, MO), and the competitive α4β2 antagonist DHβE (Sigma, St. Louis, MO) were dissolved in saline and injected SC (mecamylamine, 1 mg/kg; DHβE, 3 mg/kg) or intraperitoneally (MLA; 6mg/kg, IP) 10 m before nicotine (or saline for control experiments). The D1 receptor antagonist SCH 23390 (0.03 and 0.01 mg/kg) and D2 antagonist eticlopride (0.015 and 0.0075 mg/kg; Sigma, St. Louis, MO) were dissolved in saline and administered SC 15 m prior to nicotine. The 5-HT_{2C} receptor agonists Ro60-0175 (0.6 mg/kg; Tocris Bioscience, Ellisville, MO) and lorcaserin (0.6 mg/kg; NPS Pharmaceuticals, Toronto, Canada) were dissolved in sterile saline and injected (SC) 10 m prior to nicotine. M100907, a 5-HT_{2A} antagonist, was dissolved in 25 mM acetic acid and 0.3% Tween80 saline solution and pH balanced to ~7.2 and injected (0.5 mg/kg, SC) 30 m before nicotine. The α4β2 partial agonist varenicline (1 mg/kg, Toronto Research Chemicals, Toronto, Canada) was dissolved in saline and injected (SC) 30 m prior to nicotine. The DA and NE reuptake inhibitor Bupropion (10 mg/kg and 30 mg/kg; Toronto Research Chemicals, Toronto, ON) was dissolved in saline and administered (IP) 30 min before nicotine. The μ-opioid antagonist naltrexone was dissolved in saline and injected (2 mg/kg, SC) 30 m prior to nicotine.

6 Data Analyses

Statistical analyses were conducted with the statistical software program SPSS version 15. Three-way, mixed-model Analyses of Variance (ANOVAs) were used to compare groups of animals exposed to saline or nicotine during conditioning (between-subjects variable) on responding on the reinforced (CR) and unreinforced (NCR) levers (within-subjects variable) under the influence of saline or an acute injection of nicotine (within-subjects variable). When the effects of various pharmaceutical agents on nicotine-enhanced responding for conditioned reinforcement were examined, a 3-way ANOVA was utilized. Lever (CR vs. NCR), Nicotine (saline or nicotine), and Drug dose served as the within-subjects variables. Tukey's Post-Hoc analyses were performed on pairwise comparisons. Greenhouse Geisser corrections for degrees of freedom were used for any violations of sphericity. Detailed descriptions of the statistical analyses for each experiment are described in Chapters 3-5.

Chapter 3
Nicotine-Induced Enhancement of Responding for Conditioned Reinforcement: Role of Prior Nicotine Exposure and α4β2 Nicotinic Receptors

Abstract

Stimuli associated with nicotine can become motivationally significant and may play a role in tobacco dependence. Previous work indicates that nicotine enhances responding for a conditioned reinforcer. These studies examined the effects of prior exposure to nicotine on responding for a conditioned reinforcer, persistence of this response, and the role of α4β2 or α7 nicotinic receptor subtypes. Water deprived rats were given 13 Pavlovian conditioning sessions where a light/tone conditioned stimulus (CS) was paired with the delivery of water. Then, rats were presented with two levers; one delivered the CS as a reinforcer, and the other was inactive. Experiments examined the effect of nicotine administered prior to Pavlovian conditioning sessions on approach behaviour during CS presentations, operant responding for the conditioned reinforcer in the presence and absence of nicotine, and the persistence of responding for the conditioned reinforcer. The effects of nicotinic acetylcholine receptor (nAChR) antagonism with mecamylamine and α4β2 or α7 nAChR antagonism with dihydro-beta-erythroidine (DHβE) or methyllycaconitine (MLA) on nicotine-enhanced responding for conditioned reinforcement were examined. Nicotine enhanced approach behavior during CS presentations and potentiated operant responding for conditioned reinforcement, an effect sensitized as a result of nicotine exposure during conditioning. Responding for conditioned reinforcement and its potentiation by nicotine was stable over multiple tests. Enhanced responding for the conditioned reinforcer induced by nicotine was blocked by mecamylamine and DHβE, but not MLA. These studies suggest that nicotine enhances Pavlovian discriminated approach and shows sensitized nicotine-induced enhancements in responding for a conditioned reinforcer, an effect depending on α4β2 nAChRs.

Adapted From: **Guy, E. G.** & Fletcher, P. J. (2013). Nicotine-induced enhancement of responding for conditioned reinforcement in rats: Role of prior nicotine exposure and α4β2 nicotinic receptors. *Psychopharmacology*, *225*(2), 429-40.

1 Introduction

Tobacco dependence is considered to be primarily due to the psychoactive effects of nicotine (CDC, 2012; Chiamulera, 2005). Like other psychomotor stimulants, nicotine stimulates locomotor activity (Domino, 2001) and has rewarding and reinforcing effects as measured through conditioned place preference and drug self-administration procedures (Corrigall & Coen, 1989; Laviolette & van der Kooy, 2003a, b; Le Foll & Goldberg, 2005). However, it has been argued that the primary reinforcing effects of nicotine itself are fairly weak compared to other drugs of abuse, such as cocaine or heroin (Caggiula et al., 2002; Palmatier et al., 2007a) and that reinforcement efficacy is dependent on drug-environment interactions (Palmatier et al. 2007a; Chaudhri et al., 2006). Like other psychomotor stimulants, nicotine can enhance responding for cues that function as conditioned reinforcers because of their association with primary rewards (Olausson et al., 2004a, b). Thus, smoking behaviors may be difficult to curb because nicotine enhances the motivating properties of cues associated with obtaining rewards and this encourages further reward-seeking, including that of tobacco. Therefore, it is of value to understand the behavioural and neuropharmacological mechanisms by which nicotine enhances responding for conditioned reinforcers.

The acquisition of responding for conditioned reinforcers can easily be measured. One widely used procedure for measuring the motivating properties of conditioned reinforcers involves first training animals to associate a conditioned stimulus (CS) with a primary reward. After this Pavlovian association has been learned, the CS acquires motivational significance and a test phase is implemented in which animals can make a novel operant response reinforced by the CS, now termed a conditioned reinforcer. Importantly, this acquisition of a new response procedure provides a clear measure of the motivating and attractive nature of reward-associated cues because that response is not associated with any primary reinforcement (Everitt et al., 2008).

Responding for conditioned reinforcement critically depends on the mesolimbic dopamine (DA) system including the ventral tegmental area (VTA) and nucleus accumbens (NAc; Parkinson et al., 1999; Taylor & Robbins, 1984). Nicotine increases DA release (Imperato et al., 1986; Ferrari et al., 2001) primarily through two nicotinic cholinergic receptor subtypes: those that contain α7 subunits and heteromeric receptors with α4β2 subunits (Ortells & Barrantes 2011; Schilstrom et al., 2003). These two receptors are hypothesized to be differentially involved in

nicotine reward. The α7-containing receptors enhance mesolimbic DA transmission associated with primary reinforcement while the α4β2 NAChRs are heavily involved in not only primary reinforcement, but also sensitization of the response stimulating effects of nicotine after repeated exposure (Laviolette & Van der Kooy, 2004; Brunzell et al., 2006; Fowler et al., 2008; Kenny & Markou, 2006; Markou & Paterson, 2001; Ortells & Barrantes, 2011). In addition, current pharmacological interventions to alleviate smoking withdrawal symptoms and cravings (e.g., varenicline) target one or more of these receptor subtypes (George & O'Malley, 2004) and modify neural activity in response to smoking cues in humans (Franklin et al., 2011). Thus, nicotine could potentially alter responding for conditioned reinforcement by action at one or both of these receptor subtypes.

Olausson et al. (2003, 2004a) have shown that nicotine has two distinct effects in this test of responding for conditioned reinforcement. First, systemically administered nicotine, given daily for 15 days before the initiation of the Pavlovian conditioning phase or given immediately following each Pavlovian conditioning session, enhanced approach to the CS during the first few training sessions (Olausson et al., 2003). Second, acute injections of nicotine selectively enhanced operant responding for a conditioned reinforcer above control levels without previous exposure to nicotine surrounding the conditioning phase (Olausson et al., 2004a).

What is not clear from this previous work is whether the effects of nicotine to enhance Pavlovian approach behavior and to potentiate responding for a conditioned reinforcement (Olausson et al. 2003, 2004a) interact. All previous work has examined the effects of nicotine on either approach behavior or responding for conditioned reinforcement. The aim of the first experiment was to examine how nicotine administered just prior to Pavlovian conditioning sessions affected subsequent responding for conditioned reinforcement in the presence of nicotine. Based on previous findings that some other responses to nicotine become sensitized (Olausson, et al., 1999; Olausson et al., 2004b; Vezina, et al., 2007), we expected that the ability of nicotine to enhance responding for conditioned reinforcement would be greater in rats previously exposed to nicotine. This hypothesis was supported and the results of the first experiment hinted at potential differences in baseline incentive motivation as a function of nicotine exposure. However, due to the randomization of drug treatments across test days in that experiment, it was impossible to determine if true differences in baseline responding for conditioned reinforcement under saline conditions exist. Thus, a second experiment examined whether nicotine exposure prior to

Pavlovian conditioning sessions affected subsequent responding for conditioned reinforcement in a drug-free state. An extension of this objective was to examine whether this nicotine exposure altered the longevity of responding for the conditioned reinforcer, as measured during repeated tests of responding for conditioned reinforcement. Finally, we examined the stability of nicotine-induced enhancements of responding for conditioned reinforcement by intermittently testing the effects of nicotine on responding for the conditioned reinforcer.

Given that α4β2 and α7 nicotinic receptors influence different aspects of reinforcement (Brunzell 2006; Ortells & Barrantes 2011; Markou & Paterson 2001), a third experiment examined the effects of the competitive α7-containing nicotinic antagonist methyllycaconitine (MLA), the competitive α4β2 antagonist Dihydro-β-erythroidine hydrobromide (DHβE) and the non-selective nicotinic receptor antagonist mecamylamine on nicotine-enhanced responding for conditioned reinforcement**.**

2 Methods

2.1 Animals

Male Long-Evans rats (Charles River, Quebec, Canada) weighing 225-250g upon arrival were singly housed in a temperature (~22°C) and humidity-controlled (~50-60%) vivarium on a 12 hour light/dark cycle (lights on 0700 h-off 1900 h). Food was available at all times, but water access was restricted as described below. All procedures were approved by the Centre for Addiction and Mental Heath Animal Care Committee and adhered to Canadian Tricouncil guidelines for the humane treatment of experimental animals.

2.2 Equipment

Training and testing occurred in sound-attenuated operant conditioning chambers (Med Associates, St. Albans, VT, USA) configured with two retractable levers located 6.5 cm from either side of a recessed water receptacle positioned 3 cm from the floor of the chamber. Water was delivered by a solenoid operated water dispenser. A red stimulus light was located above each retractable lever and a Sonalert sound generator and white houselight were located at the rear of the chamber opposite the water magazine.

2.3 Experimental Procedures

The test of responding for conditioned reinforcement occurs in two main phases: a Pavlovian conditioning phase in which a CS and US are paired, and an instrumental responding phase in which rats can respond for the CS (now termed a conditioned reinforcer). Prior to each experimental session, water was restricted to 1 h each day approximately 23 h prior to the start of the next session. This procedure was followed throughout the Pavlovian phase of the study. During the instrumental responding phase, drug tests were conducted 48 h apart for the first two experiments and 72 h apart for the third experiment. To minimize stress, rats were given free access to water during intervening test days and water restriction was reinstated 23 h prior to each test session to ensure subjects were in the same state of water deprivation as in the conditioning phase. Using this procedure, animals incurred some initial weight loss at the onset of water restriction (about 10% of body weight), but steadily gained weight throughout the experiment. Free water access during intervening test days had no effect on later responding for conditioned reinforcement.

2.3.1 Pavlovian Conditioning

During this phase, the response levers were retracted and animals were subjected to 13, daily sessions consisting of 30 pairings of a 5 s light/tone CS followed immediately by the presentation of 0.05 mL of tap water (US) on a random time (RT) 60 s schedule of reinforcement. The houselights were turned on at the beginning of the session. CS presentations consisted of a 5 s illumination of the two red stimulus lights with the houselights turned off and a 2.9 kHz, 85 dB tone stimulus presented during the last 0.5 s of the light presentation. Sessions lasted 30 min on average.

2.3.2 Instrumental Responding for Conditioned Reinforcement

On the day before the first test day, rats were placed in the boxes with both levers present. Pressing the left lever delivered the CS, according to a random -ratio (RR) 2 schedule. No water was delivered. Pressing the right lever had no programmed consequences. Once the animal had responded ten times on the active lever, the levers were retracted and the session terminated. This session ensured that all animals had sampled the active lever prior to testing and to

minimize any potential confounding effects due to novelty of the levers. Tests of responding for the conditioned reinforcer were carried out in 40 min sessions. During testing sessions both levers were inserted into the boxes at the start of the sessions. Responses on the left lever delivered the CS (now a conditioned reinforcer) on a RR2 schedule. Responses on the right lever were recorded, but had no programmed consequences.

2.4 Drugs

[-]-nicotine bitartrate (Sigma, St. Louis, MO) was dissolved in saline and titrated to a pH of ~7.2. Doses are expressed as the amount of nicotine base. The non-competitive nicotinic receptor antagonist mecamylamine (Tocris Bioscience, Ellisville, MO) was dissolved in sterile saline at a concentration of 1 mg/mL. The primarily α7 nicotinic receptor antagonist MLA (Tocris Bioscience, Ellisville, MO) and the competitive α4β2 antagonist DHβE (Sigma, St. Louis, MO) were both dissolved in saline at concentrations of 6 mg/mL and 3 mg/mL, respectively.

2.5 Experiment 1: The effects of nicotine on Pavlovian approach behavior and subsequent responding for conditioned reinforcement

Twenty-four rats were randomly assigned to two treatment groups, one to receive nicotine during the Pavlovian conditioning phase (nicotine group; n =12) and one to receive saline (saline group; n =12). Prior to training, both groups received two injections, 24 h apart, of either nicotine or saline (subcutaneous-SC) according to their group designation, in the home cage to accustom them to the injection procedure and the subjective effects of nicotine. During the Pavlovian conditioning phase, rats received either nicotine (0.4 mg/kg, SC) or saline 5 min prior to each Pavlovian training session. During these sessions, the number of nosepokes into the water magazine was recorded for each 5 s CS presentation, and for each 5 s period immediately preceding the CS. The total number of nosepokes for each session was also recorded. To assess instrumental responding for the conditioned reinforcer, each rat was tested in four sessions separated by 48 h in which injections of saline, 0.1, 0.2 or 0.4 mg/kg nicotine (SC) injections preceded placement in the conditioned reinforcement testing chambers by 5 min. The numbers of responses on the lever delivering the conditioned reinforcer (CR lever) and on the inactive lever (NCR lever) were measured. Dose order was counterbalanced across all subjects using a Latin square design. Rats were not tested on the intervening days.

2.6 Experiment 2: The effects of prior nicotine exposure on baseline responding for conditioned reinforcement, persistence of responding for conditioned reinforcement, and the stability of nicotine-induced enhancements in responding for conditioned reinforcement.

Two new groups of rats (n =10 each) underwent the same injection habituation and Pavlovian conditioning procedure as described in experiment 1. To assess whether prior nicotine exposure altered baseline responding for conditioned reinforcement, all rats were first tested for the acquisition of responding for the conditioned reinforcer following injection with saline. Then, to explore whether there were any differences in sensitivity to the response-stimulating effects of nicotine, groups were tested 48 h later following a 0.2 mg/kg nicotine injection, followed 48 h later by a 0.4 mg/kg nicotine injection. Next, we examined whether repeated testing diminished responding for the conditioned reinforcer over time, and whether this measure of incentive motivation showed a differential pattern of persistence as a function of nicotine history during the Pavlovian conditioning phase. Animals were exposed to 7 consecutive tests in which no pretreatments were administered. Finally, to examine the stability of enhanced responding for conditioned reinforcement induced by nicotine animals were re-exposed to two nicotine (0.4 mg/kg) test days interspersed by a saline challenge, each separated by 48 h.

2.7 Experiment 3: Effects of mecamylamine, DhβE, or MLA on nicotine-potentiated responding for conditioned reinforcement.

In the third experiment, water-deprived rats (n = 32) were habituated to injection procedures and trained to associate the light-tone CS with the presentation of water in 13 daily sessions, as in the previous two experiments. All rats were injected SC with 0.4 mg/kg of nicotine 5 min prior to these sessions. Following training, animals were separated into three groups, matched for their magazine approach responding during CS presentations. These three groups were used to separately test the effects of three nAChR antagonists: the broad-spectrum nAChR antagonist mecamylamine (n = 12; 1 mg/kg, SC), the predominantly α7 nAChR antagonist MLA (n = 10; 6 mg/kg, intraperitoneal-IP), and the α4β2 nAChR antagonist DHβE (n = 10; SC, 3 mg/kg SC) on responding for conditioned reinforcement in the presence of nicotine (0.4 mg/kg, SC) or saline. Receptor antagonists were administered 10 min prior to nicotine or saline, which were injected 5

min before test sessions. Each rat was tested under the 4 possible drug combinations of antagonist or vehicle, and nicotine or saline. The order of treatments was determined from Latin squares with 72 h intervening between treatments. Doses, the interval between treatments, and routes of administration were based on previous reports indicating sufficient wash-out periods and efficacy in blocking other effects of nicotine with minimal effects on locomotor activity (Grottick et al., 2000; Markou & Paterson, 2001; Lof et al., 2010).

Finally, to determine if nicotine's effect to enhance responding for conditioned reinforcement remained intact when animals were not water-deprived, two additional test days were conducted using a random subset of 20 of the original 30 animals. Ten animals remained on the same water-deprivation schedule as before while the other 10 had free access to water. The effects of nicotine (0.4 mg/kg) or saline injections, administered in counterbalanced order 72 h apart, on responding for the conditioned reinforcer were assessed in these two groups.

2.8 Data Analysis

Statistical analyses were conducted using SPSS version 15.0. For Experiments 1 and 2, the number of nosepoke responses made during the 5s CS periods and the 5s pre-CS (PCS) periods in the Pavlovian phase were expressed as a proportion of the total number of nosepokes per session (Burton et al. 2010). These data were then analyzed using a three-way, mixed-model ANOVA with the Session number and Response type (CS/PCS) as within-subjects factors and Group (nicotine/saline) as the between-subjects factor. Data for the conditioned reinforcer test phases were also analyzed with a three-way, mixed-model ANOVA, with Nicotine dose and Lever (CR/NCR) as within-subjects factors and Group (saline/nicotine) as the between-subjects factor. Data for the repeated testing phase in Experiment 2 were analyzed with a three-way, mixed-model ANOVA using Test Day and Lever as the within-subjects factors and Group as the between-subjects factor. For experiment 3, the conditioned reinforcement test data were analyzed using separate three-way ANOVAs for each antagonist group (Mecamylamine/MLA/DHβE) with Lever (active/inactive lever), Nicotine treatment (saline/nicotine), and Antagonist pretreatment (saline/antagonist) as the independent variables. Violations of sphericity were corrected for using a Greenhouse-Geisser correction for appropriate degrees of freedom. Post-hoc pairwise comparisons utilized a Tukey's HSD

procedure or a Games-Howell procedure for unequal variance, where appropriate, to fix family-wise error rates at $\alpha = .05$.

3 Results

3.1 Experiment 1: The effects of nicotine on Pavlovian approach behavior and subsequent responding for conditioned reinforcement

3.1.1 Pavlovian Approach

As shown in Figure 1a, both groups exhibited discriminated approach to the water magazine across the 13 sessions, with animals responding proportionally more during the 5 s light/tone cue than during the 5 s preceding the onset of the CS (main effect of Response type, $F(1, 22) = 123.57$, $p < 0.001$). This pattern of behavior increased over sessions (Response type x Session interaction, $F(12, 264) = 22.17$, $p < 0.001$). The two groups differed in their pattern of discriminated approach behavior (main effect of Group, $F(1, 22) = 8.05$, $p = 0.01$; Response type x Group interaction, $F(1,22) = 9.52$, $p = 0.005$; Figure 1a). Post hoc analyses indicated that animals in the nicotine group exhibited proportionally higher levels of responding during the CS periods compared to saline-treated controls ($p < 0.05$). The numbers of responses during CS and PCS periods, averaged over the last 3 conditioning days, for saline and nicotine exposed groups are shown in Table 1. Analysis of variance revealed a significant Response x Group interaction, $F(1, 22) = 4.59$, $p = 0.043$ indicating that responding during CS, but not PCS, periods was higher for the nicotine exposed animals. The total number of responses was not significantly different between the two groups.

3.1.2 Responding for Conditioned Reinforcement

Data for this experiment are shown in Figures 1b and c. Both groups showed higher responding on the CR vs. NCR lever as shown by a significant main effect of Lever ($F(1, 22) = 77.43$, $p < 0.001$). The ANOVA indicated no overall effect of Nicotine dose on responding ($F(3, 66) = 0.96$, $p = 0.419$) nor a Nicotine dose x lever interaction ($F(3, 66) = 1.26$, $p = 0.294$). However, as there were differences between the nicotine-naive and nicotine-exposed animals in Pavlovian approach behavior, *a priori* planned comparisons using a Tukey's HSD procedure to correct for type 1 error inflations were used to analyze the effect of nicotine on responding for conditioned

reinforcement. As shown in Figures 1b and 1c, 0.4 mg/kg nicotine significantly enhanced responding on the CR lever compared to the saline condition in the nicotine pre-exposed group ($p < .05$), but not in the saline pre-exposed group.

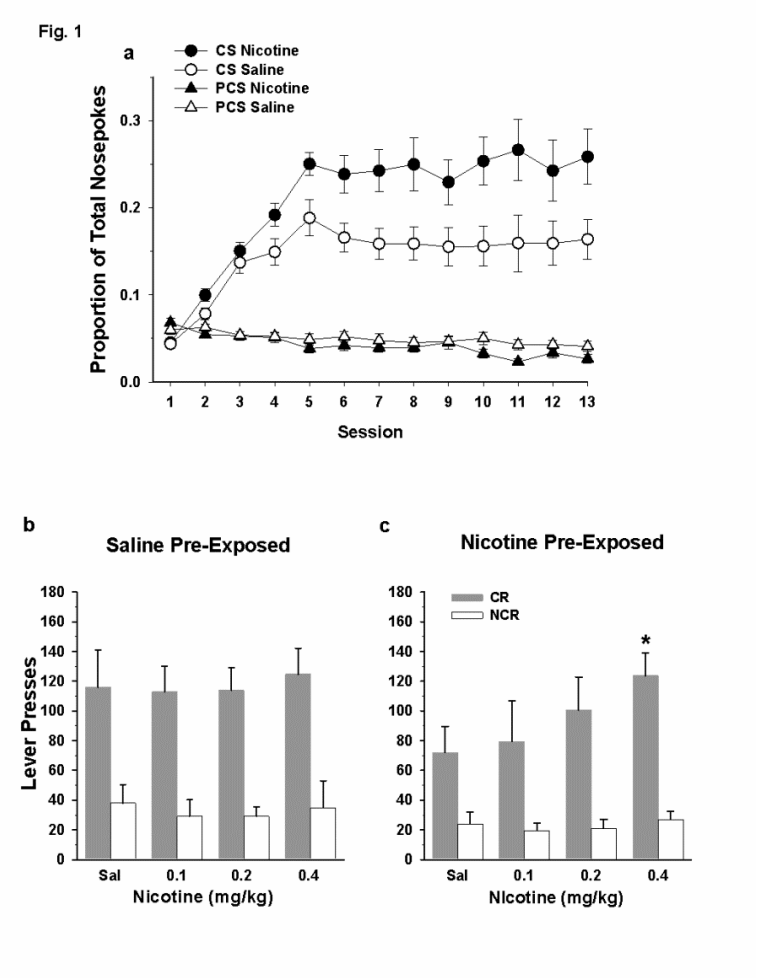

Fig. 1. a. All animal groups exhibited discriminated nosepoke behavior (nicotine CS-●; saline CS-○; nicotine NCS- ▲; saline NCS- Δ). Those animals that received nicotine injections prior to placement in the behavioral chambers exhibited a larger proportion of nosepoke activity during CS presentations compared to those that received saline prior to Pavlovian trials. **b.** Nicotine, at all doses tested, had no significant effect on CR responding in animals that received saline during Pavlovian training. **c**. In animals that received nicotine injections just prior to Pavlovian trials, the 0.4 mg/kg dose of nicotine significantly elevated CR responses.

* $p<0.05$ compared to Sal condition

Table 1.

The Effect of Nicotine on Total Nosepoke Response Behavior

Group	CS Responses	PCS Responses	Total Responses
Exp. 1			
Saline	94.06 ± 17.40	27.39 ± 6.05	601.97 ± 77.48
Nicotine	120.40 ± 22.10*	12.94. ± 3.40	433.69 ± 49.74
Exp. 2			
Saline	75.93 ± 17.56	24.70 ± 8.68	518.03 ± 103.32
Nicotine	108.07 ± 19.49*	19.97 ± 4.08	440.47 ± 42.99

Nicotine enhanced nosepoke behavior during CS presentations without altering overall nosepoke behavior. The values depicted in the table are the means ± SEM from the last three days of Pavlovian approach training, when responding was stable.
* indicates a significant enhancement in CS responding for nicotine, compared to saline ($p < 0.05$).

3.2 Experiment 2: The effects of prior nicotine exposure on baseline responding for conditioned reinforcement, persistence of responding for conditioned reinforcement, and the stability of nicotine-induced enhancements in responding for conditioned reinforcement.

3.2.1 Discriminated Approach Behavior

Again, all animals displayed discriminated approach behavior to the magazine, as indicated by a higher proportion of nosepoking during CS periods versus PCS periods (Figure 2a, main effect of Response type, $F(1, 18) = 103.31$, $p < 0.001$) which increased over sessions (Response type x Session interaction, $F(12, 216) = 17.77$, $p < 0.001$) As in Experiment 1, animals also differed by training group in their pattern of nosepoke responding percentages during the CS and PCS periods (main effect of Group, $F(1, 18) = 7.69$, $p = 0.013$; Response type x Group interaction $F(1, 18) = 6.58$, $p = 0.019$). Post hoc analyses indicated that the nicotine group again exhibited significantly higher levels of approach to the magazine during CS presentations compared to the saline group ($p < 0.05$).

A similar pattern of behavior was observed using absolute numbers of CS and PCS period responses, averaged over the last 3 conditioning days (see Table 1). Analysis of variance revealed a significant Response x Group interaction, $F(1, 18) = 4.99$, $p = 0.038$) indicating that responding during CS, but not PCS, periods was higher for the nicotine exposed animals. The total number of responses was not significantly different between the two groups.

3.2.2 Responding for Conditioned Reinforcement

No differences emerged between groups in the test of baseline incentive motivation for conditioned reinforcement (Figures 2b and 2c; $p > 0.05$). Planned pairwise comparisons of the subsequent two nicotine challenge sessions indicated that only animals exposed to nicotine prior to Pavlovian conditioning trials responded more on the CR lever following nicotine injections at the 0.2 and 0.4 mg/kg doses compared to the saline test day (Figures 2b and 2c; *p-values* < 0.05).

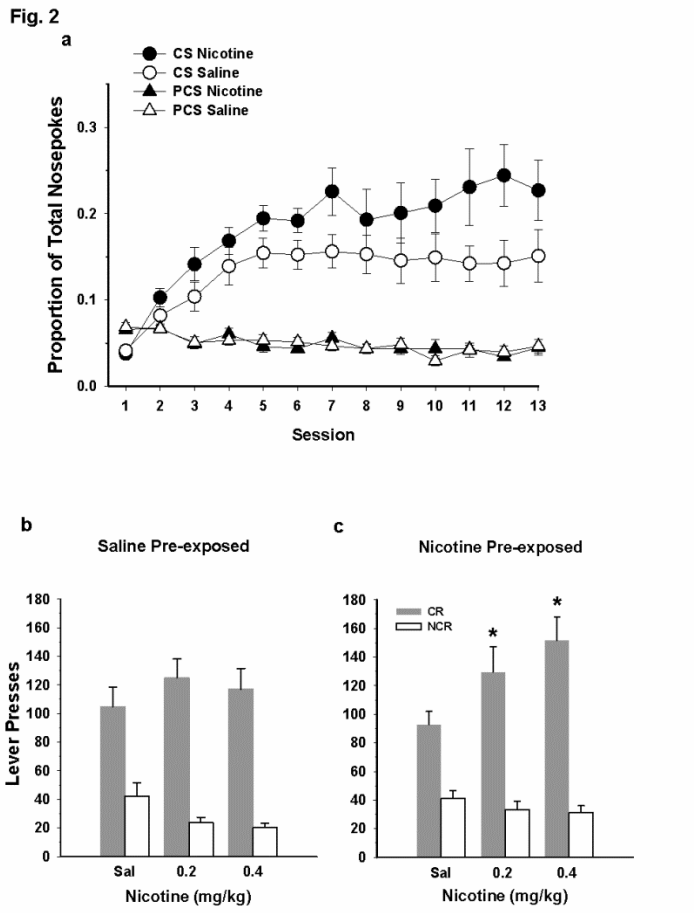

Fig. 2. a. All animals exhibited discriminated approach behavior (nicotine CS-●; saline CS-○; nicotine NCS- ▲; saline NCS- Δ. Animals that received nicotine injections during the Pavlovian training period exhibited a greater proportion of nosepoke behavior during the CS compared to animals that received saline (*Sal*) injections ($p < .05$). **b**. Nicotine failed to significantly alter responding for CR in the saline group. **c**. The 0.2 and 0.4 mg/kg nicotine doses elevated CR responding in animals previously exposed to nicotine.

* $p<0.05$ compared to Sal condition

3.2.3 Effects of Repeated Testing on Responding for Conditioned Reinforcement

Animals were then subjected to seven sessions of responding for CR to determine whether this response declined differentially as a function of prior nicotine exposure. In the following seven sessions of responding for conditioned reinforcement, responses on the CR lever diminished only slightly over the course of seven trials (Figure3; Lever x Session interaction, $F(6, 108) = 2.52$, $p = 0.026$). No significant differences between treatment groups emerged (main effect of Group, $F(1, 18) = 0.31$, $p = 0.59$; Group x Lever x Session interaction, $F(6, 108) = 1.45$, $p = 0.204$). After seven days there was still a preference for the CR versus the NCR lever (main effect of Lever, $F(1, 18) = 20.81$, $p < 0.001$).

3.2.4 Effects of Re-Testing with Nicotine on Responding for Conditioned Reinforcement

Following this repeated testing for conditioned reinforcer responding, we re-examined the ability of nicotine to enhance CR responding as a function of nicotine exposure during conditioning. A 2(Lever: CR/NCR) x 2(Group) x 3(Challenge: Saline, Nicotine challenge 1, Nicotine challenge 2) ANOVA indicated that nicotine enhanced CR responding compared to the previous saline challenge (main effect of Challenge day, $F(2, 36) = 56.83$, $p <0.001$; Challenge day x Lever interaction, $F(2, 36) = 52.31$, $p < 0.001$). Furthermore, nicotine enhanced responding for conditioned reinforcement to a greater extent in the nicotine pre-exposed group compared to the saline pre-exposed group (Figures 4a and 4b; main effect of Group, $F(1,18) = 4.60$, $p = 0.046$; Lever x Challenge day x Group, $F(2, 36) = 10.49$, $p < 0.001$). Tukey's tests confirmed that responding on the CR lever under the influence of nicotine was significantly higher ($p < 0.05$) for the animals that received nicotine in the Pavlovian training context compared to those animals that received saline.

Fig. 3

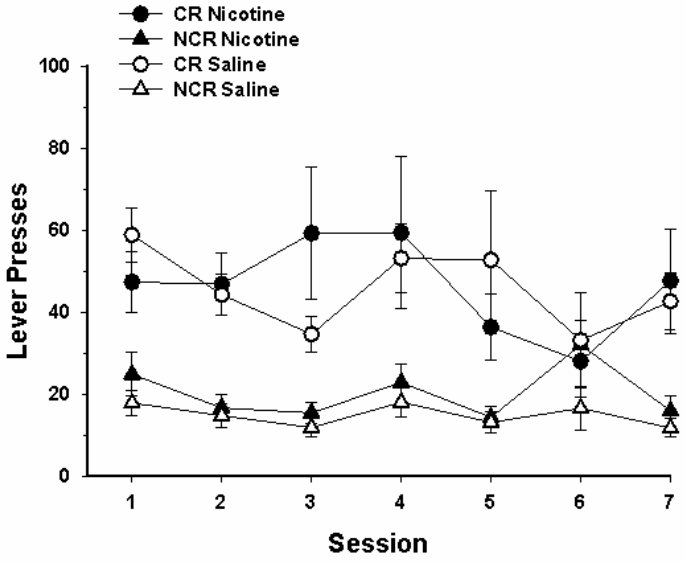

Fig. 3. Preferential responding on the CR lever versus the NCR lever persists over time ($p <$ .05), but does not differ between training groups ($p >$ .05).

Fig. 4

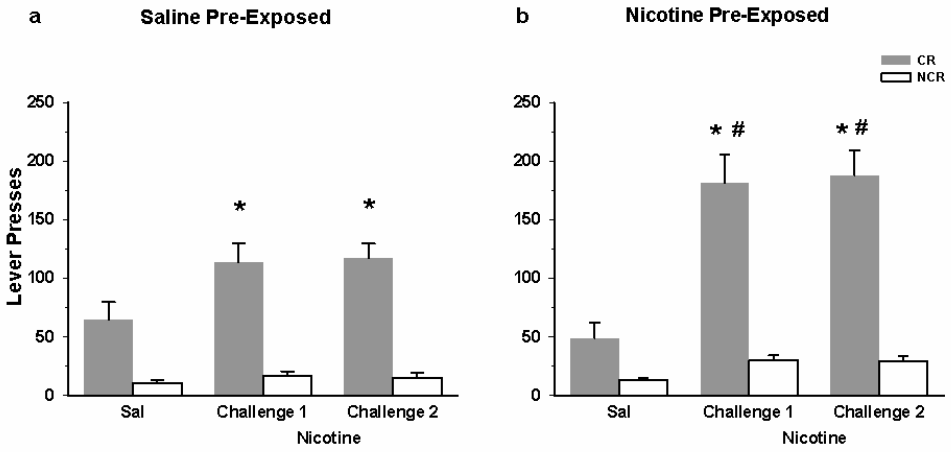

Fig. 4. a. Both nicotine challenges (*Nic Challenge*, 0.4 mg/kg) elevated responding on the CR lever compared to a saline challenge after multiple trials in animals receiving saline injections during Pavlovian conditioning sessions ($p < .05$). **b.** A nicotine challenge (0.4 mg/kg) also elevated responding on the CR lever above saline following multiple trials in animals that received nicotine during conditioning. Furthermore, the level responding on the CR lever was above that of the saline group ($p < .05$).

*p<0.05 compared to Sal condition

# p<0.05 compared to corresponding challenge condition in Saline pre-exposed rats

3.3 Experiment 3: The Effects of Nicotinic Receptor Antagonists, Alone and in Combination with Nicotine, on Responding for Conditioned Reinforcement

After Pavlovian training, all animals exhibited discriminated nosepoke responding at similar levels as in the prior experiments. The percentage of overall responding during the CS periods was significantly higher than during the PCS periods (main effect of discrimination, $F(1, 29) = 146.34$, $p < 0.001$) and discrimination increased over test sessions (response type x day interaction, $F(12, 372) = 31.07$, $p < 0.001$; data not shown).

As shown in Figure 5a, animals in the Mecamylamine group exhibited a preference for the CR lever (main effect of lever, $F(1, 11) = 38.27$, $p < 0.001$) and nicotine increased this response (main effect of Nicotine treatment, $F(1,11) = 8.97$, $p = 0.012$). There was a significant Lever x Nicotine treatment x Antagonist pretreatment interaction ($F(1,11) = 14.07$, $p = 0.003$), which can be accounted for by the fact that the Nicotine x Antagonist interaction was significant for CR responses ($F(1,11) = 11.34$, $p = 0.006$), but not NCR responses ($F(1,11) = 0.36$, $p > 0.05$). Post-hoc pairwise comparisons confirmed that the nicotine-enhanced CR response was reduced to saline CR levels by mecamylamine ($p < 0.05$).

As depicted in Figure 5b, animals in the DHβE group also exhibited an increase in responding under nicotine and a preference for responding on the CR lever (main effect of Lever, $F(1, 9) = 10.47$, $p = 0.01$; main effect of Nicotine treatment, $F(1,9) = 6.11$, $p = 0.035$). While there was not a significant three-way interaction (Lever x Antagonist pretreatment x Nicotine treatment interaction, $F(1, 9) = 1.06$, $p > 0.05$), examination of the lower-order effects did reveal a selective enhancement in CR lever responding by nicotine (Lever x Nicotine treatment interaction, $F(1,9) = 13.00$, $p = 0.006$). DHβE reduced responding on the CR lever (Lever x Antagonist pretreatment interaction, $F(1,9) = 6.13$, $p = 0.035$) and this effect was selective to nicotine-enhanced CR responding (Antagonist x Nicotine treatment interaction, $F(1,9) = 8.48$, $p = 0.017$). Tukey's HSD post-hoc analyses confirmed that DHβE blunted nicotine-induced CR responding only and this pretreatment had no effect on NCR responding or CR responding following a saline injection (p-values < 0.05).

Figure 5c shows the effect of the α7 selective nAChR antagonist MLA on responding for conditioned reinforcement. Responses were greater on the CR lever and nicotine effectively

potentiated this response pattern (main effect of Lever, $F(1,9) = 67.13$, $p < 0.001$; main effect of Nicotine treatment, $F(1, 9) = 9.64$, $p = .013$). Again, nicotine selectively enhanced CR responding (Lever x Nicotine treatment interaction, $F(1,9) = 16.89$, $p = 0.003$). MLA had no effect on overall responding or the ability of nicotine to enhance responding for CR (main effect of Antagonist pretreatment, $F(1,9) = 2.51$, $p > 0.05$; Antagonist pretreatment x Nicotine treatment interaction, $F(1,9) = 0.17$, $p > 0.05$). Post-hoc tests confirmed that MLA pretreatment had no effect on saline CR responses, nicotine-enhanced CR, or non-reinforced lever responding under any condition ($p > 0.05$).

Finally, as shown in Fig 5d, nicotine enhanced responding for CR in both water-deprived and non-deprived groups (main effect of Nicotine, $F(1,18) = 6.74$, $p = 0.02$; Nicotine x Lever interaction, $F(1, 18) = 8.67$, $p = 0.01$) and the pattern of enhanced responding did not differ between conditions (main effect of Condition, $F(1, 18) = 0.78$, $p = 0.39$; Lever x Nicotine x Condition interaction, $F(1, 18) = 0.97$, $p = 0.33$).

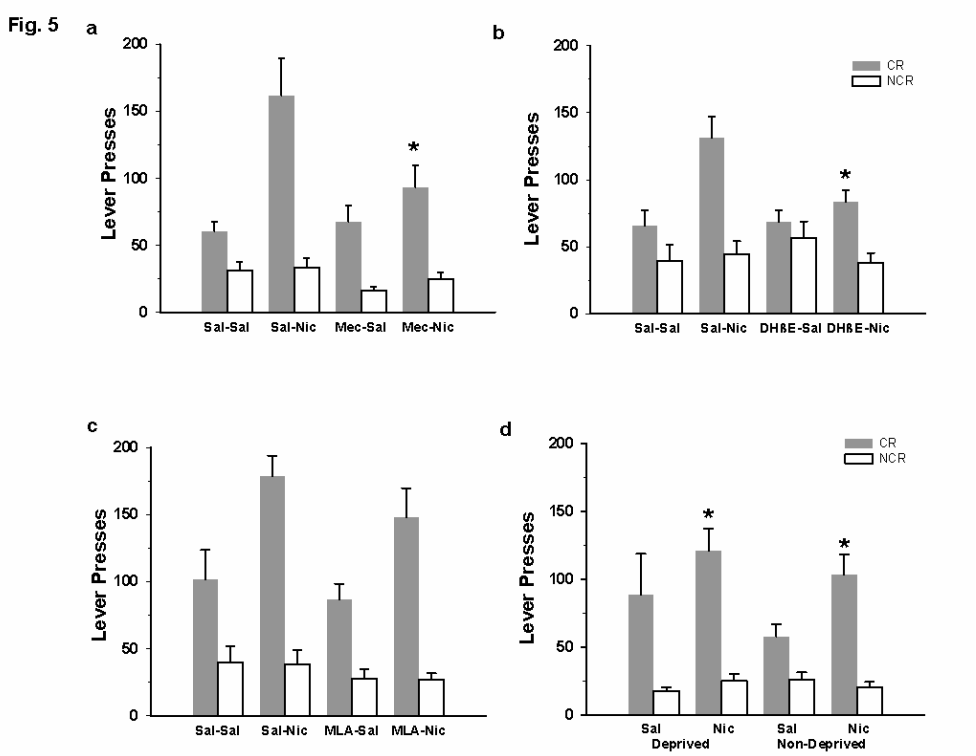

Fig. 5. Antagonist treatment conditions (*Mec/DH*βE/*MLA)* are represented before the dash (–) and the administration of nicotine (*Nic*) or saline (*Sal)* is indicated after the – for the relevant bar graphs. Nicotine enhanced responding for conditioned reinforcement. **a.** The broad-spectrum nicotinic receptor antagonist blocked nicotine-induced increases in CR responding ($p < .05$), but did not affect responding in the saline condition ($p > .05$). * Identifies a significant decrease in nicotine-enhanced responding for CR ($p < .05$). **b.** Likewise, the α4β2 nAChR antagonist DHβE blocked nicotine-induced increases in responding on the CR lever ($p < .05$) without altering baseline responding levels ($p > .05$). * Identifies a significant decrease in nicotine-enhanced responding for CR ($p < .05$). **c.** The preferential α7-containing nicotinic receptor antagonist MLA had no effect on responding for CR under both nicotine and saline conditions. **d.** Nicotine enhanced responding conditioned reinforcement in both non-deprived and thirsty animals. * indicates a significant increase in CR responding compared to saline ($p < 0.05$).

4 Discussion

These experiments found that nicotine injected prior to Pavlovian conditioning sessions enhanced discriminated approach behavior to a reward magazine, as measured by an increased proportion of nosepokes during the CS period compared to the saline condition. Subsequently, nicotine also enhanced responding for conditioned reinforcement, but this effect seemed initially dependent on prior nicotine exposure. Experiment 2 showed that responding for conditioned reinforcement is stable over repeated tests, and that the ability of nicotine to enhance responding for conditioned reinforcement does not diminish with repeated tests. Additionally, Experiment 2 suggests that enhanced responding for conditioned reinforcement by nicotine may show sensitization following repeated exposure to the conditioned reinforcement test procedure. Finally, Experiment 3 identified stimulation of α4β2 nAChRs as a primary mechanism for this effect of nicotine to increase responding for conditioned reinforcement.

The results from Experiment 1 and 2 generally agree with previous research showing that repeated nicotine administration prior to the initiation of, or immediately following, Pavlovian conditioning sessions enhances approach behavior to the reward magazine during the CS period (Olausson et al., 2003). The results also replicate previous findings that nicotine enhances operant responding for conditioned reinforcement (Olausson et al., 2004a, b). However, the results of Experiment 1 differ from these previous findings in several important ways.

First, we showed that the effect of nicotine to enhance discriminated approach behavior only emerged after several sessions. This finding differs from the report by Olausson et al. (2003), where the effect of nicotine occurred in just the first three training sessions. This difference may stem from temporal differences in nicotine administration. In our study, nicotine was administered just prior to Pavlovian conditioning sessions, rather than after sessions or prior to the entire conditioning phase (Olausson et al., 2003). Therefore these rats experienced the CS-US associations while under the influence of nicotine, and this may be the reason for enhanced approach behaviour in the presence of the CS. Whether this effect simply reflects nicotine-induced hyperactivity (Clarke & Kumar, 1983) in the presence of reward-related cues, or a more specific action on reward-related selective attention and learning is not clear. It is notable that overall nosepoke behavior was unaltered by nicotine administration (Table 1), thus the effect of nicotine to enhance responding was selective to CS periods and therefore likely not due to

generalized hyperactivity. Under certain experimental conditions, nicotine enhances attention processing (Grottick & Higgins, 2000), and perhaps this action may also contribute to the effect of nicotine to increase approach behavior during the CS presentations. Overall, there are now several reports that nicotine increases approach behavior to stimuli that predict reward availability. Understanding the psychological and behavioral processes underlying this effect is an important goal for future work.

Second, the results of Experiment 1 and the first part of Experiment 2 demonstrate that nicotine administration during Pavlovian conditioning sessions is necessary for animals to show an initial nicotine-induced enhancement in responding for conditioned reinforcement. This result differs from Olausson et al. (2004a), who found that nicotine enhanced responding for conditioned reinforcement in drug-naïve animals. This discrepancy may be due to the substantially different levels of responding for conditioned reinforcement after saline injections between the various experiments, with responding in our animals being 2-3 times higher than in previous work (Olausson 2004a). The use of Long-Evans rats, as opposed to Sprague Dawley rats, may account for this difference because Long-Evans rats may exhibit more exploratory behavior, particularly rearing (Padilla, Douglas, Shumake, & Gonzalez-Lima, 2009), and thus be more inclined towards higher rates of instrumental behavior. Further evidence that the baseline level of conditioned reinforcement responding contributes to the expression of the effect of nicotine on CR responding comes from experiment 2. Here it was found that nicotine did enhance responding for conditioned reinforcement in non-nicotine pre-exposed animals, but only after their basal CR response rate had declined as a result of more extended testing. Thus, in experiment 1 and the first part of experiment 2 the initial high level of responding under saline treatment may have masked an enhancing effect of nicotine.

The results of Experiment 1 suggested a slight reduction in responding for conditioned reinforcement under saline treatment for nicotine-exposed animals compared to saline-exposed rats. Since the saline tests were counterbalanced across the four test sessions, one aim of Experiment 2 was to assess whether true differences in initial responding for the conditioned reinforcer exist between nicotine-exposed and nicotine-naive animals, which could be indicative of a possible state-dependent influence over acquisition of responding for conditioned reinforcement. However, no differences in baseline responding for conditioned reinforcement were found using the new experimental design, as would be expected if nicotine-exposure during

Pavlovian conditioning induced some type of state-dependent learning. Consistent with the results of Experiment 1, an acute injection of nicotine enhanced responding for conditioned reinforcement in the nicotine–exposed rats but not the control rats. To examine whether the saline exposed and nicotine exposed groups differed in their persistence of responding for conditioned reinforcement, animals underwent seven test sessions without any pretreatments. While responding for conditioned reinforcement was lower than on the first saline test day, response patterns remained stable and the conditioned reinforcer remained an effective reinforcer across all sessions for both groups. No differences in CR responding emerged between groups in these trials. When rats were re-tested following injections of nicotine or saline, responding for the conditioned reinforcer was enhanced by nicotine in both groups. This effect of nicotine was higher for the group that received nicotine during Pavlovian sessions and perhaps is indicative of a sensitization-like effect of nicotine on responding for conditioned reinforcement.

Prior studies have shown that nicotine enhances acquisition of responding for visual stimuli even when they have not directly been associated with primary reward (Liu et al., 2007; Palmatier et al., 2007a, c). This process could also contribute to the response enhancing effect of nicotine, and to the persistence of this responding observed in the present experiments. However, numerous studies using conditioned reinforcement procedures have shown that the light/tone CS does not support novel instrumental responding when explicitly unpaired with a primary reinforcer during Pavlovian conditioning (Beninger & Phillips, 1980; Burton et al., 2011; Taylor & Robbins, 1984). Thus, in the present studies the CS likely acquired motivational significance through pairings with primary reinforcement. Nevertheless, both processes are consistent with the notion that nicotine persistently enhances the motivational attraction to non-pharmacological reinforcers.

Experiments 1 and 2, together with previous results, consistently show that nicotine enhances responding for conditioned reinforcement. Experiment 3 was designed to identify which nAChR is important for mediating this effect. The broad spectrum antagonist mecamylamine blunted the effect of nicotine on CR lever responding without affecting responding on the NCR lever or CR responding in the saline condition. This profile likely indicates selective diminution of the nicotine-induced enhancement of the motivational properties of the CS by mecamylamine and not an inhibition of locomotor activity. Using more selective nAChR antagonists, these studies

identified the α4β2 nicotinic receptor as the primary contributor of this effect because DHβE, but not MLA, blocked nicotine-enhanced responding for conditioned reinforcement.

In addition to blocking the effect of nicotine, the competitive α4β2 nAChR antagonist DHβE itself also abolished preferential responding for conditioned reinforcement, perhaps due to competition with the binding of endogenous ACh. This effect was seemingly rescued by nicotine administration prior to the tests of responding for conditioned reinforcement. Thus, while rats treated with DHβE alone did not show a preference for the CR versus NCR lever, responses obtained under treatment with DHβE and nicotine were not different from the control condition. The ability of DHβE to block nicotine-enhanced responding for conditioned reinforcement is in accord with prior findings that β2 knockout mice failed to show enhanced responding for conditioned reinforcement following repeated exposure to nicotine (Brunzell et al., 2006). In contrast to Lof et al. (2010), we did not find a role for α7-containing nAChRs in mediating responding for conditioned reinfrocement in the present study. This again may be due to a difference in baseline levels of responding, as responding for conditioned reinforcement in the saline condition was markedly higher in our experiment and thus may have been resistant to response decrements due to α7-containing receptor blockade at the doses used in this study. Additionally, this discrepancy may be due to differences in the type of primary reinforcer (water vs. sucrose), or to differences in the Pavlovian training condition as our animals had repeated exposure to nicotine during conditioning, which was not the case for the Lof et al. (2010) study.

Despite this inconsistency, the differential effects of the selective nicotinic receptor antagonists on responding for conditioned reinforcement are consistent with other findings. For example α4β2, but not α7 nicotinic receptors, are primarily involved in changes in mesolimbic reward pathway neurotransmission measured by locomotor sensitization and intravenous nicotine self-administration (Grottick et al., 2000). Additionally, this specific nAChR subtype is involved in mediating nicotine-induced responding for a visual stimulus (Liu et al. 2007), nicotine-induced conditioned place preference (Walters et al., 2006), nicotine-induced responding for conditioned reinforcement in mice, and enhanced efficacy of rewarding brain stimulation (Brunzell et al., 2006; Kenny & Markou, 2006). Indeed, it has been hypothesized that the low-affinity α7 receptors, located on glutamatergic terminals in the VTA, are primarily involved in facilitating the initial phasic "burst" of DA associated with the primary reinforcing effects of nicotine upon acute exposure (Laviolette & van der Kooy, 2003b; Laviolette & van der Kooy, 2004; Markou &

Paterson, 2001; Mameli-Engvall et al., 2006, Schilstrom et al., 2003; Ortells & Barrantes, 2011). In contrast, the rapidly desensitizing α4β2 receptors are thought to be involved in modulating VTA GABA neurotransmission to control the pattern of dopaminergic cell burst firing (Laviolette & van der Kooy, 2004; Schilstrom et al., 2003), which is associated with attributing incentive value to reward-predictive cues (Roitman et al., 2004). Thus, it is in line with this evidence that α4β2 nAChRs were found to be involved in the response-potentiating effects of nicotine in responding for a cue previously associated with primary reinforcement.

These studies add to accumulating evidence suggesting that nicotine dependence is, at least partially, due to the ability of nicotine to enhance the motivating properties, or incentive salience, of reward stimuli (Chaudhri et al., 2006; Kenny & Markou , 2006; Palmatier et al., 2007a; Palmatier et al., 2007b; Robinson & Berridge, 1993). These data also indicate that previous nicotine exposure sensitizes nicotine-induced enhancements in motivated behaviors elicited by reward-predictive cues, and that this effect remains robust over time in the absence of primary reward. Thus, individuals may be encouraged to persist in nicotine use by such a reliable reinforcement-enhancing action of nicotine. Additionally, this study identified the α4β2 nicotinic receptor as the mediator of nicotine-induced enhancements in incentive motivation, as measured by the conditioned reinforcement paradigm, adding to evidence that changes in α4β2 nicotinic receptor function are primarily involved in nicotine-induced alterations in reward circuits that contribute to nicotine dependence (Brunzell et al., 2006; Corrigall et al., 1994; Kenney & Gould, 2008; Kenny & Markou, 2006; Ortells and Barrantes, 2011). This further indicates the importance of this receptor subtype for the development of pharmaceutical interventions (e.g., varenicline) to attenuate tobacco cravings.

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Chapter 4
The Effects of Nicotine Exposure During Pavlovian Conditioning in Rats on Several Measures of Incentive Motivation for a Conditioned Stimulus Paired with Water

Abstract

Nicotine enhances approach toward and operant responding for conditioned stimuli (CSs), but the effect of exposure during different phases of Pavlovian incentive learning on these measures remains to be determined. These studies examined the effects of administering nicotine early, late, or throughout Pavlovian conditioning trials on discriminated approach behavior, nicotine-enhanced responding for conditioned reinforcement, extinction, and the reinstatement of responding for conditioned reinforcement. We also tested the effect of nicotine on approach to a lever-CS in a Pavlovian autoshaping procedure, and for this CS to serve as a conditioned reinforcer. Thirsty rats were exposed to 13 conditioning sessions where a light/tone CS was paired with the delivery of water. Nicotine was administered either prior to the first or last 7 sessions, or throughout the entire conditioning procedure. Responding for conditioned reinforcement, extinction, and the reinstatement of responding by the stimulus and nicotine were compared across exposure groups. Separately, the effects of nicotine on conditioned approach toward a lever-CS during autoshaping, and responding for that CS as a conditioned reinforcer, were examined. Nicotine exposure was necessary for nicotine-enhanced responding for conditioned reinforcement and the ability of nicotine and the stimulus to additively reinstate responding on the reinforced lever. Nicotine increased contacts with a lever-CS during autoshaping, and removal of nicotine abolished this effect. Prior nicotine exposure was necessary for nicotine-enhanced responding reinforced by the lever. In conclusion, enhancements in the motivating properties of CSs by nicotine occur independently from duration and timing effects of nicotine exposure during conditioning.

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1 Introduction

Nicotine reinforcement is influenced, in part, by nicotine enhancing the motivational properties of reward-related stimuli (Caggiula, Donny, White, Chaudhri, Booth, Gharib, Hoffman, Perkins, & Sved, 2001; Chaudhri, Caggiula, et al., 2006; Chaudhri et al., 2007; Liu, Palmatier, Caggiula, & Donny, 2007; Jones, Raiff, & Dallery, 2010; Palmatier et al., 2007). These conditioned stimuli (CSs) can bias attention, and reinforce continued tobacco consumption in humans; contributing to nicotine dependence and relapse (Franklin et al., 2011; Freeman, Morgan, Beesley, & Curran, 2012; Rose, Behm, Westman, & Johnson, 2001). It has been argued (Balfour, Wright, Benwell, & Birrell, 2000; Caggiula et al., 2000) that the conditioned reinforcing properties of smoking-associated CSs are at least as important for nicotine reinforcement as the primary reinforcement derived from nicotine itself, an assertion supported by evidence in both human and animal studies of nicotine reinforcement (Balfour et al., 2000; Caggiula et al., 2001; Rose et al., 2001).

The interaction between nicotine and CSs on reinforcement processes can be studied in rats using a behavioral test that measures the acquisition of a new operant response for a conditioned reinforcer (Mackintosh, 1974). In this test, during an initial Pavlovian conditioning phase a CS is associated with a primary reinforcer (i.e., unconditioned stimulus, US), such as water or food. Then, in a second phase, the animal can make a novel operant response for subsequent presentations of the CS, now serving as a conditioned reinforcer. Nicotine has two effects in this test. During the Pavlovian phase, nicotine enhances approach behavior to the location of primary reward delivery in the presence of the CS (Guy & Fletcher, 2013; Olausson, Jentsch, & Taylor, 2003). Subsequently, during the operant conditioning phase, nicotine enhances responding for that CS as a conditioned reinforcer, an effect that persists over multiple tests (Guy & Fletcher, 2013; Olausson, Jentsch, & Taylor, 2004a, 2004b).

During the Pavlovian conditioning phase, discriminated approach behavior during CS presentations increases rapidly during the initial trials, and then stabilizes (Guy & Fletcher, 2013; Olausson et al., 2003). Presumably this change in rate of approach behavior is a reflection of learning the association between CS-US. Olausson et al. (2003) showed that nicotine increased head entries in the reward receptacle in the presence of the CS during the initial conditioning trials. We also found enhanced approach behavior during these early trials, but the effect seemed

to persist throughout the conditioning phase. It is possible that the effects of nicotine to enhance approach behavior to a reward delivery receptacle in the presence of a CS may vary depending on whether it is injected during the initial acquisition phase, or once the CS-US associations have been formed. Since the ability of the CS to function as conditioned reinforcement is presumably dependent on the nature of the CS-US association, the timing of nicotine injections during Pavlovian conditioning may also alter the capacity of the CS to serve as a conditioned reinforcer. To test these possibilities, Experiment 1 compared the effects of nicotine injections administered throughout the Pavlovian conditioning phase with those resulting from nicotine administered in the early conditioning trials, during the acquisition phase, or later, during the maintenance phase. We measured receptacle approach in the presence of the CS during Pavlovian conditioning. Then, in the operant conditioning phase, we examined responding for the conditioned reinforcer and the potentiation of this response after an acute nicotine challenge.

In humans, nicotine associated CSs may enhance subjective "cravings" (Franklin et al., 2011), which in turn can trigger relapse to drug-seeking. In animals, extinguished nicotine self-administration can be reinstated by priming injections of nicotine (Chiamulera, Borgo, Falchetto, Valerio, & Tessari, 1996) and by response-contingent presentations of nicotine-associated CSs (Le Sage, Burroughs, Dufek, Keyler, & Pentel, 2004; Liu, Caggiula, Yee, Nobuta, Poland, & Pechnick, 2006). In the latter case, such CSs may be functioning as conditioned reinforcers. Given the potentially large role of conditioned reinforcing stimuli to maintaining addiction-related behaviors (Balfour et al., 2000; Caggiula et al., 2001; Rose et al., 2001), and the interaction between nicotine and conditioned reinforcers (Olausson et al., 2004a,b; Guy & Fletcher, 2013), we measured reinstatement of extinguished operant responding for that reinforcer. Based on previous findings (Le Sage et al., 2004; Caggiula et al., 2001), we predicted that reinstatement would be greatest when nicotine was given in conjunction with a conditioned reinforcer. We also examined whether such reinstatement would vary as a function of the timing and duration of nicotine exposure during the initial Pavlovian conditioning phase.

Experiment 1 demonstrated a role for nicotine exposure in the expression of nicotine-induced increases in responding for a conditioned reinforcer, and reinstatement of that response after it had been extinguished. However, nicotine did not enhance discriminated approach in the reward receptacle in the presence of the CS during the Pavlovian conditioning phase. In this procedure, the only behavior measured during CS presentations was approach to the location of the primary

reward. It is possible that nicotine may have enhanced incentive learning in these animals, but that this effect may not have been apparent in this measure. In fact, Pavlovian-conditioning based incentive learning could be expressed via a number of different behaviors (Flagel, Watson, Robinson, & Akil, 2007; Silva, Timberlake, & Gont, 1998). For example, Silva et al. (1998) showed that animals may engage with reward-predictive stimuli as part of a "generalized search" response to the CS. Other studies have shown that individual animals differ in their conditioned approach behavior; some preferentially approach the CS itself (sign-tracking), while others approach the reward location (goal-tracking) during CS presentations (Flagel et al., 2007; Flagel, Akil, & Robinson, 2010; Flagel, Clark, Robinson, Mayo, Czuj, Willuhn, Akers et al., 2011). Therefore, in a second study we measured the effect of nicotine on approach behaviors to both the CS itself (henceforth referred to as sign-tracking behavior) and to the water receptacle during CS presentations (goal-tracking behavior), using a Pavlovian autoshaping procedure (Flagel et al., 2007) adapted for use in water-deprived animals. Similar to Experiment 1, we varied the timing and duration of nicotine exposure during the Pavlovian autoshaping phase, and subsequently tested the ability of the CS used during autoshaping to serve as a conditioned reinforcer, as well as the effect of acute nicotine on this response. Together, these studies provide a characterization of the effect of nicotine exposure on Pavlovian incentive learning in two different behavioral tests, and whether any such effects translate to differences in nicotine-enhanced motivation for a conditioned reinforcer.

2 Methods

2.1 Animals

Male Long-Evans rats (Charles River, Quebec, Canada) weighing 225-250g upon arrival were singly housed in a temperature (~22°C) and humidity-controlled (~50-60%) vivarium on a 12 hour light/dark cycle (lights on 0700 h-off 1900 h). Food was available at all times, but water access was restricted as described below. All procedures were approved by the Centre for Addiction and Mental Heath Animal Care Committee and adhered to Canadian Tricouncil guidelines for the humane treatment of experimental animals.

2.2 Experiment 1A: Effects of Nicotine Administered during Different Phases of Pavlovian Conditioning on Approach Behavior and Responding for a Conditioned Reinforcer

2.2.1 Pavlovian Conditioning

Testing occurred in sound-attenuated operant conditioning chambers (Med Associates, St. Albans, VT, USA) containing two retractable levers located 6.5 cm on either side of a recessed water delivery receptacle positioned 3 cm from the floor of the chamber. An infrared photocell detector in the receptacle recorded head entries. A stimulus light was located above each response lever. The day prior to beginning Pavlovian conditioning sessions, animals were restricted to 1 h of water access per day and remained water-restricted throughout conditioning and testing procedures. Each conditioning session consisted of 30 pairings of a 5 s CS followed immediately by the presentation of 0.05 mL of tap water (US) on a random time (RT) 60 s schedule of reinforcement. Sessions lasted on average for 30 min. The CS was a 5 s illumination of the two red stimulus lights with the houselight off and a 2.9 kHz, 85 dB tone stimulus presented during the last 0.5 s of the light presentation. Rats were randomly assigned to one of four groups. Group 1 (Saline Controls, n = 10) was administered saline injections prior to each Pavlovian conditioning session. Group 2 (Nicotine Throughout, n = 9) received nicotine injections (0.4 mg/kg, SC) just prior to each Pavlovian training session. Group 3 (Nicotine Early, n = 10) received nicotine injections prior to the first seven Pavlovian conditioning sessions and saline for the remaining sessions. Group 4 (Nicotine Late, n = 10) received saline injections on sessions 1-6 and nicotine injections prior to sessions 7-13.

2.2.2 Responding for a Conditioned Reinforcer

During tests of responding for a conditioned reinforcer, 2 levers were inserted into the chambers. Responding on one lever (CR lever) resulted in presentation of the CS, in the absence of the water reward, on a RR2 schedule of reinforcement so that each response had a 0.5 probability of reinforcement. Responses on the other lever, (NCR) had no programmed consequences. All rats underwent 2 counterbalanced test sessions, spaced 72 h apart; one session was preceded by a saline injection and one was preceded by a nicotine (0.4 mg/kg, SC) injection.

2.2.3 Responding During Extinction Conditions and Reinstatement Tests

Seven, 40 min daily extinction sessions were conducted in which responses on both levers had no consequences. Next, rats were injected with saline or nicotine and placed in the chambers where lever responses were recorded, but not reinforced. The order of saline and nicotine treatment was counterbalanced with 72 h between tests. Responding was extinguished in between these two test sessions. After four further daily extinction sessions, a second set of reinstatement tests, administered 72 h apart, was given following injections with saline or nicotine (0.4 mg/kg). This time, responses on the CR lever were paired with conditioned reinforcer presentations.

2.3 Experiment 1B: Effect of Nicotine on Responding for a Stimulus Explicitly Unpaired with Water

This experiment examined whether responding for the light/tone stimulus in Experiment 1A, and its potentiation by nicotine, was due to the fact that the stimulus acquired conditioned reinforcing properties through pairings with the water US.

2.3.1 Unpaired Training

Rats were exposed to 13 daily sessions consisting of 30 presentations of the light/tone stimulus used as the CS in experiment 1A and 30 0.05 mL water deliveries. Both stimuli were presented pseudo-randomly, and were explicitly unpaired. Six rats received saline and 6 rats received nicotine (0.4 mg/kg) injections prior to these sessions.

2.3.2 Responding for the Light/Tone Stimulus

Two tests of operant responding for the light/tone stimulus, spaced 72 h apart, were conducted as described for Experiment 1A. Tests were preceded by saline or nicotine (0.4 mg/kg) injections.

2.4 Experiment 2: Effects of Nicotine on Goal-Tracking, Sign-Tracking, and Responding for a Conditioned Reinforcer

2.4.1 Autoshaping

The purpose of this experiment was to determine if nicotine altered approach specifically to a CS, measured over 6 daily autoshaping sessions. To match the number of injections administered to the Nicotine Throughout and Saline groups from Experiment 1, rats (n = 40) were divided into two groups and received one injection daily for 7 days prior to behavioral testing. One group received nicotine (0.4 mg/kg, SC; n = 20), and the second group received saline injections (SC; n = 20). Then, water was restricted as described in Experiment 1A.

Training took place in the same chambers as Experiment 1, but with a different configuration. A red houselight was switched on throughout the session. During each session, 25 CS-US pairings were delivered on a variable time (VT)-90 s schedule of reinforcement. The CS consisted of the insertion of the left lever into the chamber, backlight illuminated by a flush-mounted 0.6 cm high output LED light. After 8 s, the lever was retracted and 0.05 mL of tap water (the US) was delivered to the central water receptacle. Sessions took place at the same time each day and lasted on average 45 min. In all sessions, contacts with the lever-CS (sign-tracking behavior) and head entries into the water receptacle during CS presentations (goal-tracking behavior) were recorded. A lever contact was measured by closure of a microswitch, adjusted to approximately 15 g of tension. Head entries in the water receptacle in the absence of the CS were recorded separately.

Nicotine exposed animals were administered nicotine, and saline exposed rats received saline 5 min prior to the 6 Pavlovian autoshaping sessions. Over these sessions, nicotine enhanced approach to the CS (sign-tracking), but not the reward receptacle (goal-tracking). Given these results, we decided to extend the experiment and determine if this approach behavior could be modified by the removal or addition of nicotine administration in 6 additional autoshaping sessions, resembling the exposure regimen used in Experiment 1. Thus, 10 of the nicotine-exposed animals and 10 of the saline-exposed animals were switched to saline or nicotine injections prior to a further 6 autoshaping sessions. For comparison with Experiment 1, these groups were named Nicotine Early and Nicotine Late, respectively. The remaining animals continued receiving saline (Saline group) or nicotine (Nicotine Throughout group) as before.

2.4.2 Responding for a Conditioned Reinforcer

After 12 Pavlovian autoshaping sessions, all animals underwent tests of responding for the lever-CS as a conditioned reinforcer. The lever-CS was moved to the center panel of the chamber in place of the water receptacle. Two nosepoke ports were placed either side of the lever. Nosepoke responses into the reinforced (CR) port resulted in a 2 s presentation of the illuminated lever. Nosepokes into the other port (NCR) were recorded, but had no programmed consequences. Responses on the lever-CS during conditioned reinforcer presentations were also recorded. Responding for a conditioned reinforcer was measured in 40 min sessions on two consecutive days. Then, subjects were given 2 test days, separated by 48 hrs, that were preceded by counterbalanced saline or nicotine (0.4 mg/kg, SC) injections 5 mins prior to placement in the operant conditioning chambers.

2.4.3 Data Analysis

Statistical analyses were conducted using SPSS version 15.0. For the Pavlovian phase of Experiment 1, head entry responses into the reward delivery receptacle made during the 5 s CS periods and the 5 s pre-CS periods were expressed as a proportion of the total number of responses per session, as in previous reports (Burton, Nobrega, & Fletcher, 2010; Guy & Fletcher, 2013). These data were analyzed using a three-way, mixed-model ANOVA with Session number and Response type (CS/pre-CS) as within-subjects factors and Group (Nicotine Throughout, Nicotine Early, Nicotine Late, or Saline) as the between-subjects factor. Tests of responding for a conditioned reinforcer used a three-way, mixed-model ANOVA with Lever (CR/NCR) and Treatment (Nicotine/Saline) as within-subjects factors and Group as the between-subjects factor. Responding during extinction was examined with a three-way ANOVA with Lever and Extinction Day as within-subjects factors and Group as the between subjects factor. Analyses of reactivation data used a four-way ANOVA with Lever, Treatment, and Reinforcer (conditioned reinforcer present/ conditioned reinforcer absent) as within-subjects factors and Group as the between subjects factor.

For Experiment 2, Pavlovian autoshaping data were analyzed using separate ANOVAs for the two Response Types (goal-tracking/ sign-tracking). Data from the two phases (sessions 1-6 versus sessions 7-12) were analyzed separately. Session served as the within-subjects factor and

Autoshaping Group (Nicotine/Saline for the ANOVA for phase 1; Nicotine/Nicotine Early/Nicotine Late/Saline for the ANOVA for phase 2) served as the between-subjects factors.

Tests of responding for a conditioned reinforcer in Experiment 2 also used a mixed-model ANOVA with Response Type (CR port/NCR port) and Treatment (Nicotine/Saline) as within-subjects factors and Autoshaping Group (Nicotine/Nicotine Early/Nicotine Late/Saline) as the between-subjects factor. Responses on the lever itself when it was presented as a conditioned reinforcer were analyzed with a 2-way ANOVA where Treatment (Saline/Nicotine) was the within-subjects factor and Autoshaping Group was the between-subjects factor.

Violations of sphericity were corrected for using a Greenhouse-Geisser correction for appropriate degrees of freedom. Pairwise comparisons utilized Tukey's HSD or Games-Howell procedures for unequal variance, where appropriate, to fix family-wise error rates at $\alpha = .05$.

3 Results

3.1 Experiment 1A: Effect of Nicotine Administered During Different Phases of Pavlovian Conditioning on Approach Behavior and on Responding for a Conditioned Reinforcer

3.1.1 Pavlovian Approach

Figure 1 shows that all groups developed discriminated approach behavior to the water receptacle (main effect of Response type; $F(1, 35) = 177.87$, $p < .001$) with animals responding in the water receptacle more during the 5s CS periods compared to the 5s period prior to the onset of the CS. This pattern of discriminated approach increased across sessions (Response type x session interaction; $F(12, 420) = 28.89$, $p < 0.001$). The overall pattern of behavior did not differ between nicotine administration groups ($p > 0.05$).

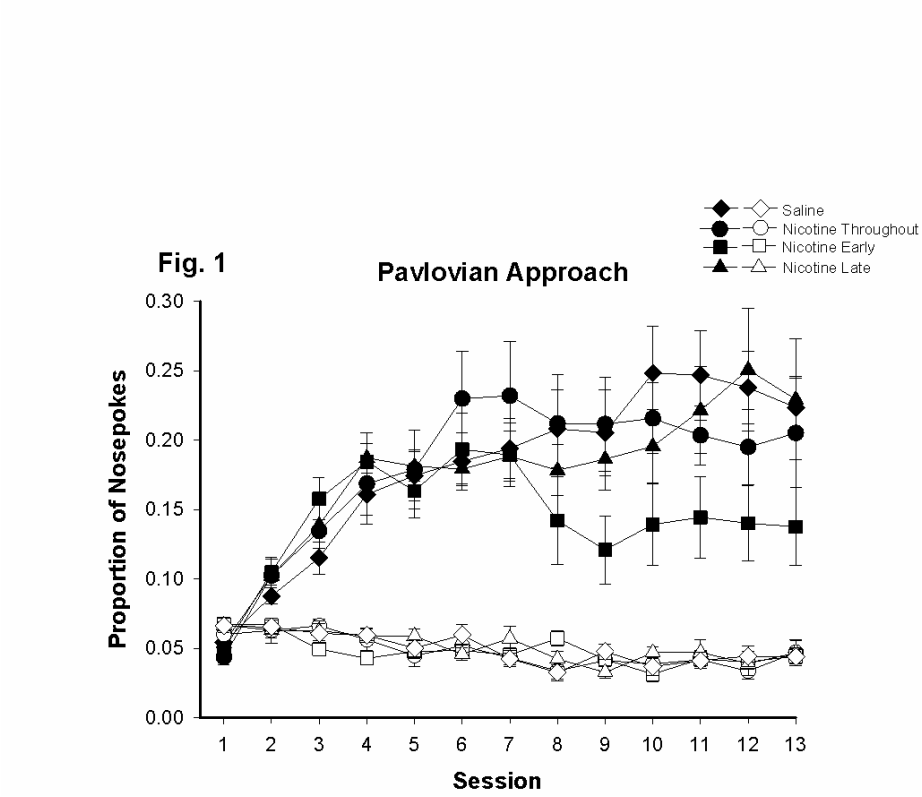

Fig. 1. This figure shows the pattern of approach behavior in response to presentations of a CS paired with water compared to the 5s prior to CS presentations (pre-CS) in groups of rats treated with nicotine throughout conditioning (Nicotine Throughout; 0.4 mg/kg; days 1-13; circles), nicotine early in conditioning (Nicotine Early; days 1-7, squares), nicotine late in conditioning (Nicotine Late; days 7-13, triangles), or saline (Saline; days 1-13; diamonds). Head entries into the water delivery receptacle were measured during the entire session, during each 5s period of CS presentations, and during the 5s immediately preceding each CS. Data points represent the mean (±SEM) proportion of total head entry activity during periods when the CS was presented (filled symbols) compared to the 5 s before the onset of the CS (pre-CS; empty symbols).

3.1.2 Test of Conditioned Reinforcement

Figure 2A shows the mean (±SEM) number of responses on the CR and NCR levers. All groups preferentially responded on the CR lever (main effect of Lever; $F(1, 35) = 154.09$, $p < 0.001$) and nicotine generally enhanced responding for a conditioned reinforcer (main effect of Treatment; $F(1, 35) = 16.684$, $p < 0.001$; Treatment x Lever interaction; $F(1, 35) = 18.86$, $p < 0.001$). Although, the Lever x Treatment x Group interaction was not significant ($p > 0.05$), we had a priori hypotheses that a history of nicotine exposure would enhance responding for a conditioned reinforcer. Pairwise comparisons of responding on the CR lever indicated that nicotine enhanced responding for a conditioned reinforcer compared to saline ($p < 0.05$), but only in those animals that experienced nicotine during the Pavlovian conditioning phase.

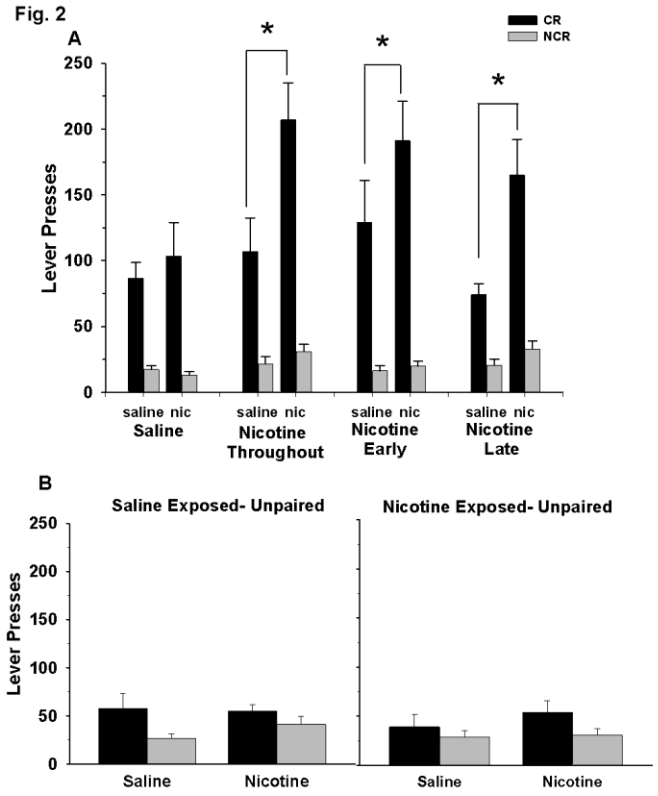

Fig. 2. Panel A shows the effects of nicotine on operant responding on the CR and NCR levers when the light/tone CS had been paired with water during the Pavlovian conditioning phase. Bars depict the mean (±SEM) number of responses on the lever that delivered conditioned reinforcement (CR, dark bars) and on the lever with no programmed consequences (NCR, grey bars). * $p < .05$ compared to corresponding saline treatment.

Panel B shows the effects of acute injection with nicotine or saline on responding on the CR and NCR levers when the light/tone CS had been explicitly unpaired with water**.** Bars represent the mean (±SEM) level of responding on CR (dark bars) and NCR levers (grey bars). Separate groups of rats had previously been treated with saline (saline exposed) or nicotine (nicotine-exposed) during the unpaired conditioning phase.

3.1.3 Extinction of Responding on the Reinforced Lever

Following removal of the conditioned reinforcer, responding diminished across days (main effect of Extinction Day; $F(5, 175) = 4.223$, $p = 0.001$, data not shown). The pattern of responding declined similarly for all groups ($p > 0.05$). The number of extinction responses, averaged over the last 3 days for each group, is shown in the first pair of bars on each panel of Figure 3.

3.1.4 The effect of Reintroducing Nicotine, or Nicotine and a Conditioned Reinforcer, on Responding on the CR Lever

Figure 3 shows that responding on the CR lever increased when nicotine, the conditioned reinforcer, or nicotine and the conditioned reinforcer were reintroduced on test sessions. As shown by the overall four way interaction, these effects of nicotine challenge and reinforcer availability differed between the Pavlovian training groups (Lever x Reinforcement x Treatment x Group interaction; $F(3, 35) = 5.14$, $p = 0.005$). This interaction was accounted for by differential 3 way interactions between Lever x Reinforcement x Treatment across the 4 Groups. Thus, the three groups exposed to nicotine during Pavlovian conditioning showed a significant 3-way interaction between the Lever x Reinforcement x Treatment (Nicotine Throughout, $F(1, 8) = 9.64$, $p = 0.02$; Nicotine Early, $F(1, 8) = 18.99$, $p = 0.002$; Nicotine Late, $F(1, 8) = 17.96$, $p = 0.002$). However, the saline exposed animals did not show this interaction ($p > 0.05$). Further decomposition of the 3-way interactions indicated that when the conditioned reinforcer and nicotine were both present, responding on the CR lever increased for each of the nicotine-exposed groups compared to responding when just the conditioned reinforcer was made available (Reinforcement x Nicotine interactions; all F-values > 8, $p < 0.03$), but not the Saline group ($p > 0.05$). Examining the main effects for each of the four groups revealed that when conditioned reinforcement again was made available, responding in general increased (all F-values > 17, $p < 0.003$). Further analyses indicated that the reintroduction of conditioned reinforcement enhanced responding on the CR lever for all groups compared to saline responding in the absence of reinforcement. However, statistical significance was observed only in the Saline and Nicotine Early groups. Nicotine enhanced overall responding in all the nicotine-exposed groups (all F-values > 20, $p < 0.002$), but not the Saline group ($p > 0.05$). Nicotine itself enhanced responding on the CR-lever in all groups, but significance ($p < 0.05$) was observed only for the Nicotine Throughout and Nicotine Late groups.

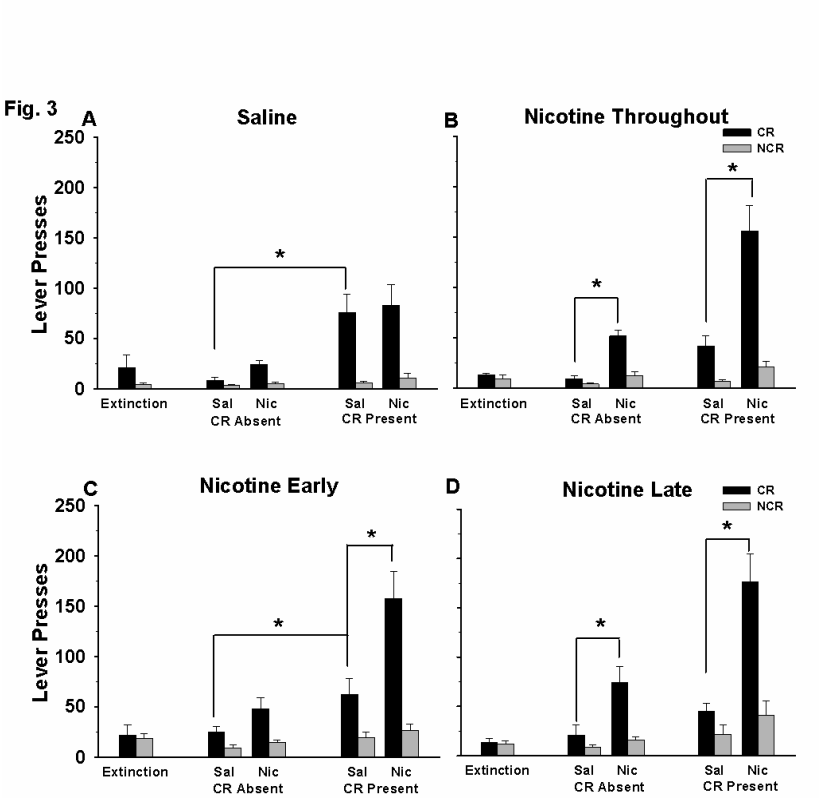

Fig. 3. The effects of nicotine history on the reinstatement of responding for conditioned reinforcement induced by nicotine, the CS, or the combination of nicotine and the CS. Bars show the mean (±SEM) number of responses on the reinforced (CR) lever (dark bars) and non-reinforced (NCR) lever (grey bars) for the Saline group (panel A), the Nicotine Throughout group (panel B), the Nicotine Early group (panel C), and the Nicotine Late group (panel D). Within each group, responding on the two levers was measured after injection with saline or nicotine, and with or without response-contingent conditioned reinforcer presentations. Average responding over the last 3 extinction days is shown for comparison. * denotes significant enhancements in responding on the CR lever ($p < 0.05$).

3.2 Experiment 1B: The effect of Nicotine on Responding for a Stimulus Explicitly Unpaired with Water

As depicted in Figure 2B, the only statistically significant effect was for the main effect of lever ($F(1, 10) = 7.25$, $p < 0.02$). Overall, responding was higher on the CR vs NCR lever. However, responding was not altered by nicotine exposure during conditioning, or acute nicotine during tests of responding for conditioned reinforcement (*p-values* > 0.05).

3.3 Experiment 2: Effects of Nicotine on Goal-tracking, Sign-tracking, and Responding for a Conditioned Reinforcer

3.3.1 Pavlovian Autoshaping Phase 1

As shown in Figure 4A, head entries in the water receptacle (goal-tracking) during CS presentations showed a slight, but significant increase over time (main effect of Session; $F(5, 175) = 6.41$, $p = 0.001$). This effect did not differ between nicotine or saline exposed groups (*p-values* > 0.05). There was also a trend for responding on the lever CS (sign-tracking) to increase across sessions (main effect of Session; $F(5, 175) = 2.58$, $p = 0.03$); responding on the lever CS (sign-tracking) was significantly higher for the nicotine exposed animals (Fig. 4C; main effect of Autoshaping Group; $F(1, 35) = 9.86$, $p = 0.003$), but the overall pattern of sign-tracking behavior did not differ between groups (*p-values* > 0.05).

3.3.2 Pavlovian Autoshaping Phase 2

Head entries into the water receptacle slightly increased over time (main effect of Session; $F(5, 180) = 2.91$, $p = 0.04$); but, as depicted in Figure 4B, this trend did not differ between the four groups ($p > 0.05$). In contrast, the four groups did differ in their overall level of sign-tracking as measured by lever responses (Figure 4D; main effect of Autoshaping Group; $F(3, 36) = 4.46$, $p = 0.01$). Tukey's Post-Hoc analyses indicated that the animals that were maintained on nicotine (Nicotine Throughout) showed higher levels of lever responding compared to the nicotine-exposed animals switched to saline (Nicotine Early) and the Saline exposed animals (*p-values* < 0.05). The pattern of sign-tracking behavior remained stable across sessions (*p-values* > 0.05).

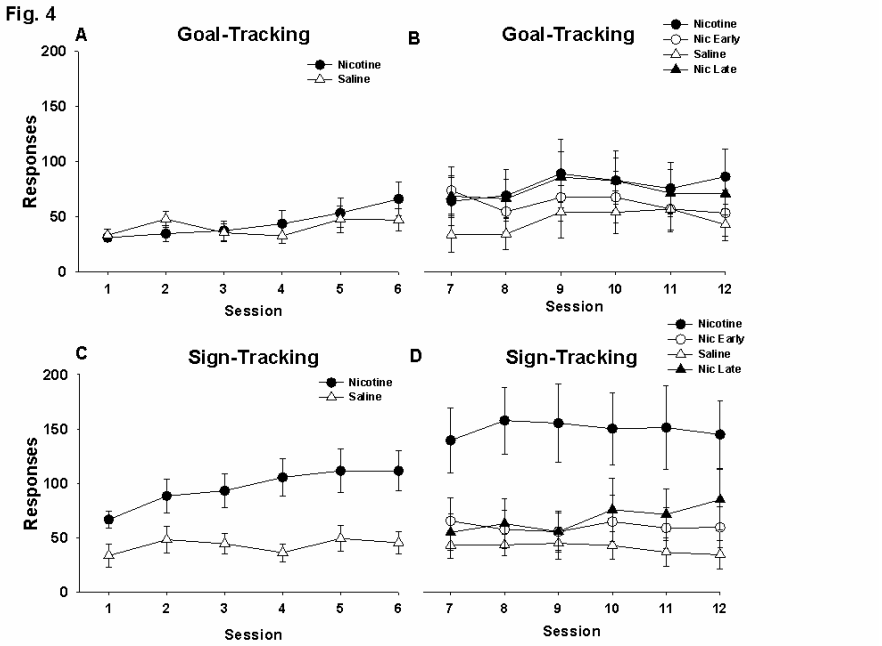

Fig. 4. The effects of nicotine on goal and sign-tracking behavior in an autosphaing task. Goal-tracking behavior was measured as the mean (±SEM) number of head entries into the water delivery receptacle during the 8s lever-CS presentations. Panel A displays the data for the Nicotine (filled circles) and Saline groups (open circles) for the first 6 autoshaping sessions. Panel B depicts the receptacle entries for the final 6 sessions, where a subset of Nicotine and Saline-exposed animals were switched to pretreatments with saline (Nicotine Early-open circles) or nicotine (Nicotine Late- filled triangles). The effects of nicotine exposure on sign-tracking behavior is displayed in panels C and D as the mean (±SEM) number of contacts with the lever-CS upon 8s presentations. Panel C displays the data for Nicotine (filled circles) and Saline groups (open circles) for the first 6 autoshaping sessions. Panel D shows sign tracking for the final 6 sessions where a subset of Nicotine and Saline-exposed animals were switched to pretreatments with saline (Nicotine Early-open circles) or nicotine (Nicotine Late- filled triangles).

3.3.3 Tests of Conditioned Reinforcement

All groups responded more in the reinforced aperture (CR) than in the unreinforced (NCR) response aperture (Fig. 5; main effect of Response Type; $F(3, 36) = 51.11$, $p < 0.001$). Overall responding was lower on day 2 than day 1, but moreso for the CR aperture (main effect of Day; $F(1, 36) = 26.26$, $p < 0.001$; Response Type x Day interaction; $F(1, 36) = 17.80$, $p < 0.001$). Responding did not differ between the four groups on either test day ($p > 0.05$).

The administration of nicotine prior to conditioned reinforcement testing resulted in increased responding in the CR aperture (Fig. 6; Response Type x Treatment interaction; $F(1, 36) = 10.39$, $p = 0.003$). Post-hoc analyses indicated that nicotine enhanced responding for a conditioned reinforcer in those animals with a history of nicotine administration during autoshaping ($p <0.05$). The animals that received saline throughout the autoshaping phase did not show this effect ($p > 0.05$).

Nicotine also increased contacts with the lever during the test for conditioned reinforcement (Fig. 7; main effect of Nicotine, $F(1, 36) = 24.92$, $p < 0.001$), but only for animals with a history of nicotine exposure (*p-values* < 0.05). Further examination of this effect indicated that animals that received nicotine during the early autoshaping trials (Nic Early) or throughout autoshaping (Nic Throughout) exhibited significantly higher lever contacts ($p < 0.05$) than animals that received saline injections (Saline) or nicotine injections in the later trials (Nic Late).

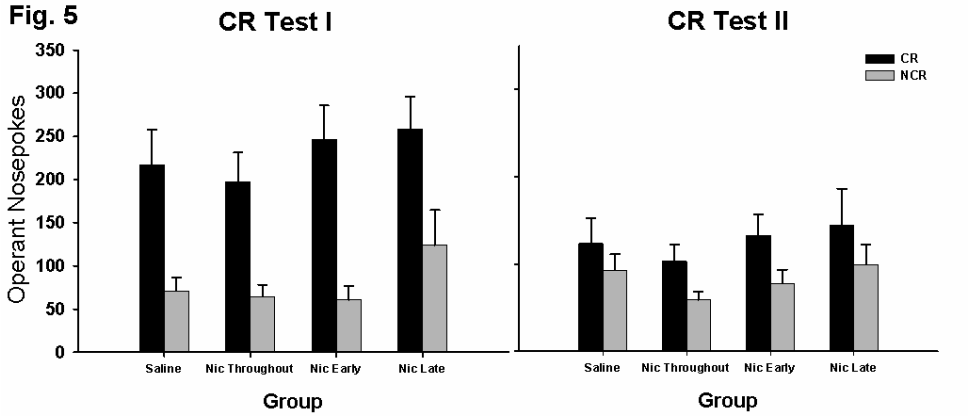

Fig. 5. Animals were tested on two occasions to determine whether the lever CS functioned as a conditioned reinforcer. In all groups rats preferred responding in the reinforced (CR) operant nosepoke aperture (dark bars) to the unreinforced (NCR) aperture (grey bars), but this effect was not altered by nicotine exposure during the Pavlovian autosphaping phase.

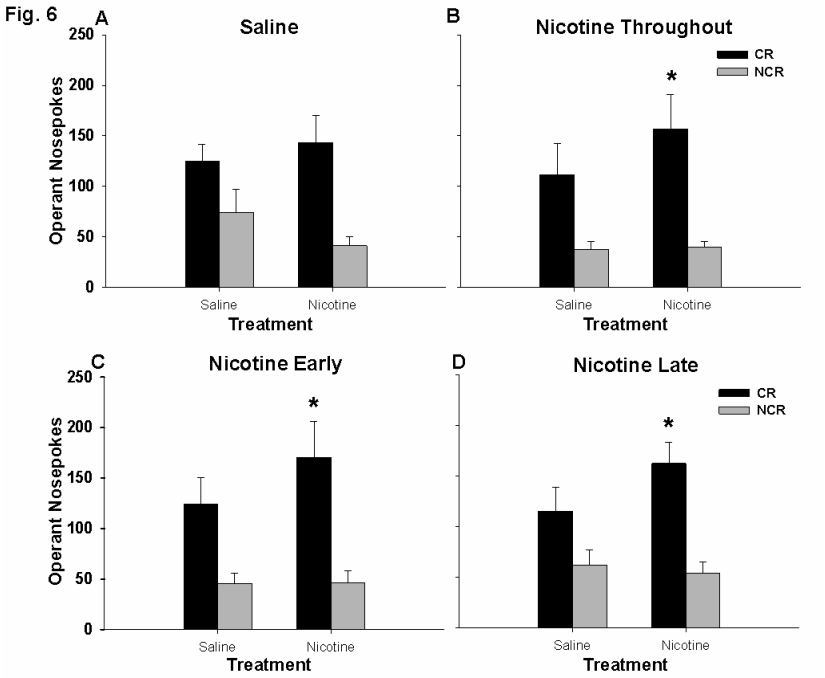

Fig. 6. The ability of nicotine to enhance responding for the lever-CS as a conditioned reinforcer depended on prior exposure to nicotine. Bars represent the mean (±SEM) level of nosepoke operant responding in the reinforced aperture (CR) compared to the aperture with no programmed consequences (NCR) for each of the four training groups. * Denotes a significant enhancement in responding in the reinforced nosepoke aperture on nicotine test sessions compared to saline ($p < .05$).

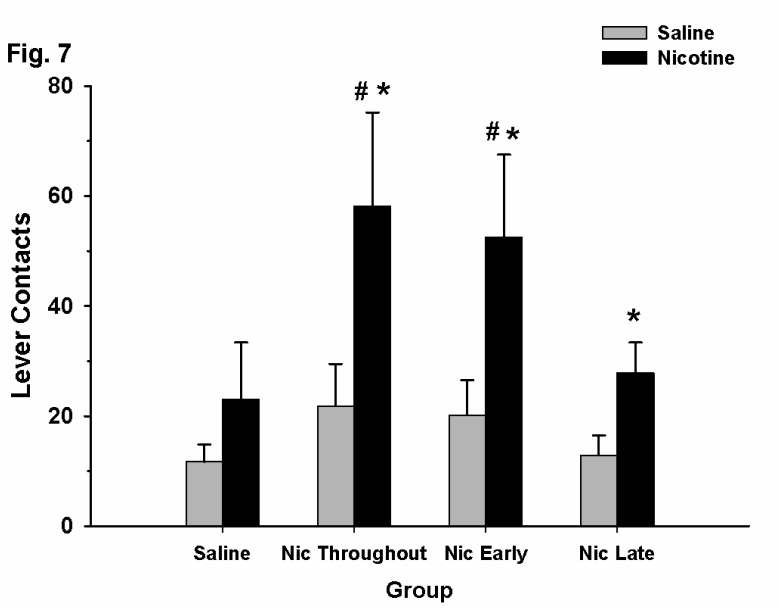

Fig. 7. The schedule of nicotine exposure during Pavlovian autoshaping affects nicotine-enhanced approach toward the CS when it serves as a conditioned reinforcer. Bars depict mean (±SEM) engagement with the lever-CS under the influence of saline (grey bars) or nicotine (dark bars) for the four different autoshaping groups. * indicates a significant enhancement in lever contacts by nicotine ($p < 0.05$). # demarks significantly higher lever contacts in the nicotine conditioned compared to the Nicotine Late and Saline groups ($p < 0.05$).

4 Discussion

These studies generated four main findings. First, in contrast to previous findings (Guy & Fletcher, 2013; Olausson et al., 2003), nicotine administered during the Pavlovian phase of a conditioned reinforcement procedure did not significantly enhance CS-elicited approach behavior to the reward-delivery receptacle. However, in a second experiment based on an autoshaping procedure, nicotine strongly enhanced contact with the CS itself during Pavlovian conditioning. Second, the CS that predicted water delivery acquired conditioned reinforcing properties in both the conditioned reinforcement and autoshaping tasks. Responding for the conditioned reinforcer was enhanced by acute injections of nicotine in nicotine-exposed animals, but this effect did not depend on the specific schedule of nicotine exposure. Third, extinguished responding was reinstated by response-contingent presentations of the conditioned reinforcer, and by priming injections with nicotine. These stimuli appeared to have at least an additive effect on reinstating responding. The effect was also dependent upon a prior history of nicotine exposure during conditioning, but again not schedule-dependent. Finally, the timing of nicotine exposure during Pavlovian autoshaping did appear to affect attraction to the CS itself during the test for conditioned reinforcement following acute nicotine injections (see Fig. 7). Animals that received nicotine during the initial autoshaping trials (Nicotine Early and Nicotine Throughout) displayed higher levels of lever contacts during the test of conditioned reinforcement. Overall, these results replicate and extend reports showing that nicotine interacts with reward-predictive cues to enhance processes related to incentive motivation.

Previously, nicotine administered prior to or throughout the entire Pavlovian conditioning phase enhanced approach to the reward-delivery receptacle when the CS was present (Guy & Fletcher, 2013; Olausson et al., 2003). In Experiment 1, we determined whether the timing of nicotine administration was critical to this effect by comparing animals receiving nicotine before each conditioning trial with those receiving nicotine before the first or last 7 days of conditioning. Unlike previous results (Guy & Fletcher, 2013; Olausson et al., 2003), nicotine administered before each conditioning session did not enhance approach during CS presentations. However, rats that received nicotine over the first 7 conditioning sessions did seem to show a reduction in the amount of discriminated approach behaviour once nicotine injections were discontinued, indicating some influence of nicotine over this response. In Experiment 2, using an autoshaping procedure in which approach to both the CS and the reinforcer location were monitored (Flagel

et al., 2007), nicotine selectively enhanced engagement with the reward-predictive illuminated lever CS without significantly altering approach to the reward-receptacle. Such sign-tracking behavior was enhanced in animals that were exposed to nicotine during the first 6 Pavlovian autoshaping trials, and the removal of nicotine resulted in a decrease in this response. However, animals that received nicotine beginning on the 6th trial (i.e., the Nicotine Late autoshaping group) did not demonstrate enhanced sign-tracking behavior. This implies that the effect of nicotine to enhance sign-tracking requires rats to experience the initial CS-US contingencies while under the influence of nicotine.

These latter results complement those of Palmatier et al. (2012) in showing a selective effect of nicotine on sign-tracking behavior. However, we did not see a long-lasting effect of elevated responding directed toward the CS when nicotine was discontinued, but instead observed decreased sign-tracking behavior. This is likely due to differences in the measures of sign-tracking behavior, the type of reinforcer used (sucrose vs. water), or a combination of both factors. Our measure of sign-tracking behavior was engagement with an illuminated lever-CS (Flagel et al., 2007), rather than head entries into a receptacle located just below the CS (Palmatier et al., 2012). Perhaps the increased physical effort of engaging in a lever response (Nicola, Taha, Kim, & Fields, 2005), compared to nosepoke responses, shows differences in sensitivity to nicotine discontinuation. In a different study, where lever responses were recorded as a measure of the reinforcing properties of a visual stimulus, discontinuing nicotine injections resulted in a similar reduction in operant responding (Palmatier et al., 2007). Regarding the type of reinforcer used, evidence from other studies indicates that nicotine is more effective in enhancing approach responses when primary reinforcement with a higher intrinsic reward value is used in conditioning procedures (Chaudhri et al., 2006; Palmatier et al., 2007; Palmatier, O'Brien, & Hall, 2012). Thus, our use of a water reinforcer, instead of sucrose (Palmatier et al., 2012), may have resulted in the drop off in sign-tracking behavior when nicotine injections were discontinued. Despite these inconsistencies, results from a number of different procedures show that nicotine can enhance Pavlovian approach behavior, but that the expression of the response may differ based on several procedural variables.

Following completion of the Pavlovian conditioning phases in both test procedures, injections of nicotine enhanced responding for the CS as a conditioned reinforcer only in animals that were exposed to nicotine during Pavlovian conditioning or autoshaping. There were no differences

between the Nicotine Early, Late, or Throughout groups in this regard. In a control experiment where the CS and water reinforcer were explicitly unpaired, animals showed a weak preference for the lever delivering the CS, and nicotine had no effect on responding for this stimulus in any group (Fig. 2B). These findings imply that the effects of nicotine observed in the operant conditioning phase of Experiment 1, and reported previously (Guy & Fletcher, 2013; Olausson et al., 2004b), reflect an enhancement by nicotine of the conditioned rewarding properties of the stimulus previously associated with water, rather than a simple nicotine-induced increase in responding for a neutral sensory stimulus (e.g. Chaudhri et al., 2006).

While the effect of nicotine to enhance responding for conditioned reinforcement was not dependent on the precise schedule of prior nicotine administration, the responses on the lever during the test for conditioned reinforcement in Experiment 2 (Fig. 7) indicated some differences between exposure groups in the attribution of salience to the CS. This response appeared to be potentiated by having received nicotine prior to the initial autoshaping sessions (i.e., sessions 1-6). It is possible that sensitization to nicotine (Vezina, McGehee, & Green, 2007), regardless of when it is administered during the Pavlovian conditioning phase, may affect the ability of nicotine to subsequently potentiate responding for a conditioned reinforcer. In contrast, approach to the lever-CS while under the influence of nicotine during the test for conditioned reinforcement may reflect differences in learned conditioned responses to presentations of the CS.

In Experiment 1, removal of the conditioned reinforcer from the test context extinguished responding on the CR lever for all groups at a similar rate. The reintroduction of both nicotine and the conditioned reinforcer enhanced responding on the CR lever over extinction levels. However, only the previously nicotine-exposed animals showed an additive enhancement of responding for conditioned reinforcement following the reintroduction of nicotine and the reinforcement after extinction (see Fig. 3). This parallels findings that reacquisition of nicotine-seeking behavior in rodents is stronger when both nicotine-associated CSs and priming injections are used (Caggiula et al., 2001; Le Sage et al., 2004). Reinstatement of extinguished responding for the conditioned reinforcer did not differ between the various pre-exposure groups, indicating that the timing of prior nicotine exposure in relation to CS-US pairings, or the number of nicotine injections, were not critical factors in determining reinstatement of responding. Again, one implication of this is that effect of nicotine to enhance reinforcement-seeking behavior in the

presence of a CS may be a sensitization effect that is not influenced by the schedule of nicotine administration during Pavlovian conditioning.

4.1 Concluding Remarks

These results add to a growing body of evidence indicating that nicotine interacts with reward-associated conditioned stimuli to alter behavior. The results from the autoshaping procedure suggest that exposure to nicotine early during incentive learning may also enhance attraction toward those reward stimuli, potentially reflecting a form of attention bias. However, results from the operant conditioning phases of these experiments suggest that a probable sensitization to the invigorating effects of nicotine enhances the conditioned reinforcing properties of reward-associated stimuli. This implies that any interactions between nicotine and the CSs during Pavlovian approach behavior may be dissociable from the ability of nicotine to enhance the reinforcing properties of these CSs in the acquisition of a new response. These results have implications for tobacco use and addiction; suggesting that a reinforcing property of nicotine, to enhance the motivational properties of reward-related stimuli (Chaudhri, Caggiula, et al., 2006; Chaudhri et al., 2007; Donny et al., 2003; Horger, Giles, & Schenk, 1992; Liu et al., 2007), can occur regardless of whether the motivational significance of such stimuli was acquired under the influence of nicotine.

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Chapter 5
Effects of Dopamine Receptor Antagonists, a 5-HT_{2C} Receptor Agonist, or a 5-HT_{2A} Receptor Antagonist on Nicotine-Induced Enhancement of Responding for Conditioned Reinforcement in Rats

Abstract

An aspect of nicotine reinforcement that may contribute to tobacco addiction is the effect of nicotine to enhance the motivational properties of reward-associated cues, or conditioned stimuli (CSs). Several studies have now shown that nicotine enhances responding for a stimulus that has been paired with a natural reinforcer. This effect of nicotine to enhance responding for a conditioned reinforcer is likely due to nicotine-induced enhancements in mesolimbic dopaminergic activity, but this has not been directly assessed. In this study, we assessed roles for dopamine (DA) D_1 or D_2 receptors, and two serotonin (5-HT) receptor subtypes known to modulate DA activity, the 5-HT_{2C} or 5-HT_{2A} subtypes, on nicotine-enhanced responding for a conditioned reinforcer. Water-restricted rats were exposed to Pavlovian conditioning sessions, where a CS was paired with water delivery. Then, in a second phase, animals were required to perform a novel, lever-pressing response for presentations of the CS as a conditioned reinforcer. Nicotine (0.4 mg/kg) enhanced responding for the conditioned reinforcer. To examine potential roles for dopamine (DA) and serotonin (5-HT) receptors in this effect, separate groups of animals were used to assess the impact of administering the D_1 receptor antagonist SCH 23390, D_2 receptor antagonist eticlopride, 5-HT_{2C} receptor agonist Ro 60-0175, or 5-HT_{2A} receptor antagonist M100907 on nicotine-enhanced responding for conditioned reinforcement. SCH 23390, eticlopride, and Ro 60-0175 all reduced responding for conditioned reinforcement, and the ability of nicotine to enhance this effect. M100907 did not alter this behavior. Together, these studies indicate that DA D1 and D2 receptors, but not 5-HT_{2A} receptors, contribute to the effect of nicotine to enhance responding for a conditioned reinforcer. This effect can also be modulated by 5-HT_{2C} receptor activation.

1 Introduction

Smoking tobacco is one of the leading causes of preventable deaths in North America (CDC, 2013). Nicotine is the major psychoactive ingredient in tobacco smoke (Stolerman & Jarvis, 1995). Current theories suggest that nicotine addiction and dependence is driven, in part, not just by the primary reinforcing effects of nicotine, but also the reinforcing effects of stimuli that are associated with nicotine use. This is supported by studies of nicotine self-administration in both animals and humans indicating that such conditioned stimuli (CSs) may be at least as powerful as nicotine itself in reinforcing nicotine-seeking and consumption (Balfour, Wright, Benwell, & Birrell, 2000; Caggiula et al., 2001; Rose, Behm, Westman, & Johnson, 2000). Preclinical studies in rodents have suggested that nicotine may also enhance the motivating properties of CSs associated with non-pharmacological rewards, such as food or water (Caggiula et al., 2001; Chaudhri et al., 2006; Christian Chiamulera, 2005). The ability of nicotine to enhance the motivating properties of these non-pharmacological CSs may also represent a critical component of nicotine reinforcement (Caggiula et al., 2001; Chiamulera, 2005).

As an example of the ability of nicotine to enhance the motivating properties of CSs, we and others have shown that nicotine potentiates responding for conditioned reinforcement (Guy & Fletcher, 2013a; Olausson, Jentsch, & Taylor, 2004a, 2004b). In these experiments, an initially motivationally neutral stimulus was paired with the delivery of water reward. Through conditioning, these CSs acquired reinforcing properties, as shown by the demonstration that animals will respond for the CS in the absence of any primary reinforcement (Mackintosh, 1975). We found that the ability of nicotine to enhance responding for conditioned reinforcement was dependent on a history of nicotine injections administered prior to daily Pavlovian conditioning sessions, and was mediated by α4β2 nicotinic acetylcholine receptors (nAChRs; Guy & Fletcher, 2013a).

Mesolimbic dopaminergic neurons projecting from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) have been implicated in responding for conditioned reinforcers. Psychomotor stimulants, such as the dopamine enhancers pipradrol and *d*-amphetamine, increase responding for conditioned reinforcement (Beninger, Hanson, & Phillips, 1980; Robbins, Watson, Gaskin, & Ennis, 1983; Robbins, 1975, 1978). Furthermore, local microinjections of d-amphetamine into the NAc enhance such responding (Taylor & Robbins, 1984). Such effects of

psychostimulant drugs on responding for conditioned reinforcement can be reversed by targeted ablation of mesolimbic DA neurons with 6-hydroxydopamine (6-OHDA; Taylor & Robbins, 1986) or by systemic injections of dopamine receptor antagonists (Ranaldi & Beninger, 1993), or the antagonism of dopamine receptors locally within the NAc (Wolterink, Phillips, Cador, Donselaar-Wolterink, Robbins, & Everitt, 1993).

The reinforcing effect of nicotine may be mediated by enhanced mesolimbic DA activity through the stimulation of cholinergic receptors in the VTA (Balfour et al., 2000; Di Chiara & Imperato, 1988; Di Chiara, 2000; Laviolette & van der Kooy, 2004; Markou, 2008). Dopaminergic activity in the VTA-NAc pathway is necessary to support nicotine self-administration (Corrigall & Coen, 1991; Corrigall, Franklin, Coen, & Clarke, 1992). Mesolimbic DA release in response to nicotine administration may reinforce nicotine-seeking behaviors by enhancing the motivating properties of nicotine-paired CSs (Caggiula et al., 2001; Balfour et al., 2000). Several studies in rodents point to the importance of these CSs in reinforcing nicotine consumption. The presence of a CS paired with nicotine delivery supports reliable self-administration behavior, and CS removal dramatically reduces responding for nicotine (Caggiula et al., 2001). Furthermore, these nicotine-associated CSs reliably reinstate self-administration behavior after extinction of the drug-taking response (Fletcher et al., 2012; LeSage, Burroughs, Dufek, Keyler, & Pentel, 2004; Liu et al., 2006), perhaps by acting as conditioned reinforcers. Supporting a role for DA in the motivating properties of these CSs, the reinstatement of nicotine-seeking behavior is reduced by systemic administration of DA receptor antagonists (Liu et al., 2011). Considering the roles for dopaminergic activity in invigorating responding for conditioned reinforcement, reinforcing nicotine administration, and the ability of nicotine CSs to reinstate nicotine-seeking behavior; it is likely that intact DA tone is also necessary for nicotine-enhanced responding for conditioned reinforcement, but this has not been directly assessed.

Other neurotransmitter systems that affect mesolimbic DA function may also interact with the effect of nicotine to enhance responding for conditioned reinforcement. For example, microinfusions of serotonin (5-HT) in the NAc block the potentiating effects of *d*-amphetamine on responding for a conditioned reinforcer (Fletcher, 1996). The 5-HT_{2A} and 5-HT_{2C} receptor subtypes respectively facilitate or blunt mesolimbic DA overflow in response to psychostimulant drug administration (Higgins & Fletcher, 2003; Navailles, De Deurwaerdère, Porras, & Spampinato, 2004; Porras, Di Matteo, Fracasso, Lucas, & Spampinato, 1999). Recent reports

demonstrate roles for these receptors in nicotine self-administration and reinstatement (Fletcher et al., 2012; Higgins et al., 2012). The reinstatement of responding on a lever previously paired with the delivery of nicotine by both a nicotine prime and a nicotine-associated CS is blocked by administering either the 5-HT_{2C} receptor agonist Ro 60-0175 or the 5-HT_{2A} receptor antagonist M100907 (Fletcher et al., 2012). Therefore, it is possible that serotonin, acting via these 5-HT receptor subtypes, may also be involved in nicotine-enhanced responding for conditioned reinforcement.

Based on this work, we hypothesized that drugs that block D_1, D_2, or 5-HT_{2A} receptors, or that stimulate 5-HT_{2C} receptors, would reduce nicotine-enhanced responding for a conditioned reinforcer. To test these predictions, thirsty rats were given Pavlovian conditioning sessions where they learned to associate a light/tone CS with the delivery of a water reinforcer. These rats received a nicotine injection prior to each of these Pavlovian sessions, since this pre-exposure is necessary to reveal the response enhancing effect of nicotine (Guy & Fletcher 2013a, b). Then, we examined the effects of systemic injections of the D_1 receptor antagonist SCH 23390, D_2 receptor antagonist eticlopride, the 5-HT_{2C} receptor agonist Ro 60-0175, and 5-HT_{2A} receptor antagonist M100907, alone and administered prior to an acute nicotine injection, in tests of responding for conditioned reinforcement.

2 Methods

2.1 Subjects

Fifty-Four male Long-Evans rats (Charles River, Quebec, Canada) weighing 225-250g upon arrival were singly housed in a temperature (~22°C) and humidity-controlled (~50-60%) vivarium on a 12 hour light/dark cycle (lights on 0700 h-off 1900 h). Food was available at all times, but water access was restricted as described below. All procedures were approved by the Centre for Addiction and Mental Health Animal Care Committee and adhered to Canadian Tricouncil guidelines for the humane treatment of experimental animals.

2.2 Apparatus

All training and testing occurred in sound-attenuated operant conditioning chambers (Med Associates, St. Albans, VT, USA) containing two retractable levers located 6.5 cm on either side of a recessed water delivery receptacle positioned 3 cm from the floor of the chamber. An

infrared photocell detector within the water receptacle recorded head entries. A red stimulus light was located above each response lever. Water was delivered by a solenoid operated water dispenser into a water receptacle. A Sonalert sound generator and white houselight were located at the rear of the chamber opposite the water delivery receptacle.

2.3 Procedures

The conditioned reinforcement test involved two main phases: a Pavlovian conditioning phase, in which a CS was paired with water; and an operant conditioning phase, in which animals could press a lever to deliver the CS, now serving as a conditioned reinforcer.

2.3.1 Pavlovian conditioning

The day prior to the initiation of Pavlovian conditioning sessions, animals in all experimental groups were restricted to 1 h of free water access and remained water-restricted throughout conditioning and testing procedures. Response levers were retracted throughout the Pavlovian conditioning phase. Each Pavlovian conditioning session consisted of 30 pairings of a 5 s CS followed immediately by the presentation of 0.05 mL of tap water (US) on a random time (RT) 60 s schedule of reinforcement. Sessions lasted on average 30 min. The CS consisted of a 5 s illumination of the two red stimulus lights located above the retracted levers equidistant on either side of the water delivery receptacle, with the houselights turned off. A 2.9 kHz, 85 dB tone stimulus was presented during the last 0.5 s of the red stimulus light presentation. Five min prior to each Pavlovian session, all rats were given an injection of 0.4 mg/kg nicotine. Rats were exposed to 13 daily sessions in total.

2.3.2 Responding for the conditioned reinforcer

During tests of responding for the conditioned reinforcer, the two retractable levers were inserted into the chambers for the first time. Responding on one lever resulted in presentation of the CS, in the absence of the water reward, on a RR2 schedule of reinforcement (i.e., each press on the reinforced lever had an approximately 0.5 probability of reinforcement). This schedule of reinforcement has been used in many previous experiments measuring responding for conditioned reinforcement, (Taylor & Robbins, 1984; Guy & Fletcher, 2013a, b; Fletcher, Korth, Robinson, & Baker, 2002; Wolterink et al., 1993). The schedule induces responding that is sensitive to drug induced increases, including those induced by nicotine (Taylor & Robbins,

1984; Guy & Fletcher, 2013a, b), and decreases in responding (Fletcher, 1996; Fletcher et al., 2002). Responses on the left lever delivered the CS as a conditioned reinforcer, and this lever is henceforth termed the CR lever. Responses on the right lever, designated the NCR lever, had no programmed consequences. The numbers of CR and NCR lever responses were recorded.

2.3.3 Responding for water

Several of the test drugs (SCH 23390, eticlopride, and Ro 60-0175) reduced responding for the conditioned reinforcer. Therefore, we also examined the impact of these treatments on responding for the primary reinforcer (water) in the same animals. Following completion of all tests of responding for conditioned reinforcement, rats were trained to respond for water according to a random-ratio schedule 2 (RR2), where on average every second lever press was reinforced by delivery of 0.05 ml water. Rats responded on the previously non-reinforced (NCR) lever for water, and no programmed CSs accompanied water delivery. Once responding had stabilized (~4 days), the effects of SCH 23390, eticlopride, or Ro 60-0175 on responding for water were examined.

2.4 Experiments

Five groups of animals were exposed to nicotine injections and CS-US pairings in the Pavlovian conditioning phase. Then, all animals underwent tests of responding for conditioned reinforcement, where separate groups were used to test the effects of SCH 23390 (Experiment 1, n = 12), eticlopride (Experiment 2, n =12), Ro 60-0175 (Experiment 3, n = 10), and M100907 (Experiment 4; two groups, n= 10 each) on responding for a conditioned reinforcer, and the ability of nicotine to increase this response. All experiments used a fully factorial design in which each animal received every dose of the appropriate test compound in combination with saline or nicotine. Drug-nicotine combinations were randomized across test days using a Latin Squares design. Test compound administration preceded saline or nicotine, and the specific interval of administration for the two treatments in each experiment are described below. Test days were separated by a minimum of 72 h. In tests of responding for water, animals were tested under each dose of the appropriate drug, with dose order selected from Latin Squares, and spaced 72h apart.

2.4.1 Experiment 1: The effects of SCH 23390 on responding for conditioned reinforcement and water

Each rat received six tests of responding for conditioned reinforcement in which SCH 23390 (0.01 and 0.03 mg/kg) or its vehicle, saline were administered 15 min prior to injections of nicotine (0.4 mg/kg) or saline. Behavioral testing began 5 min after the second injection.

On completion of the conditioned reinforcement test phase, animals were trained to respond for water. Then, on 3 test days, the effects of administering saline or SCH 23390 (0.01 mg/kg or 0.03 mg/kg) on responding for water were assessed. Tests for water responding began 20 min post-injection. Doses of SCH 23390 were selected on the basis that they reduced nicotine self-administration (Corrigall & Coen, 1991) and reinstatement of nicotine-seeking (Liu et al., 2011).

2.4.2 Experiment 2: The Effects of eticlopride on responding for conditioned reinforcement and water

Animals in this experiment each received six tests of responding for conditioned reinforcement. In these tests, saline or eticlopride (0.0015 mg/kg or 0.03 mg/kg) injections preceded the administration of a second injection of saline or nicotine (0.4 mg/kg) by 15 min. Animals were placed in the operant chambers 5 min following the second injection. Doses of the D_2 receptor antagonist eticlopride (0.0075 and 0.015 mg/kg) were selected based on reports that they reduce the reinstatement of nicotine-seeking by a nicotine-paired CS without affecting responding reinforced by food (Liu et al., 2011), and increase responding for psychomotor stimulants on simple fixed ratio schedules of reinforcement (Botly, Burton, Rizos, & Fletcher, 2008; Hubner & Moreton, 1991; Koob, Le, & Creese, 1987). Animals underwent six test days in total. Following the final operant test day, animals were trained to respond for the primary reinforcer. When responding stabilized, the effects of administering saline or eticlopride (0.0015 mg/kg or 0.03 mg/kg) 20 min prior to tests of responding for water were examined.

2.4.3 Experiment 3: The effect of Ro60-0175 on responding for conditioned reinforcement and water

Each rat underwent a total of 4 test days where injections of Ro 60-0175 (0.6 mg/kg) or saline were administered 10 min prior to an injection of saline or nicotine (0.4 mg/kg), which were administered 5 min prior to placement in the test chambers. The dose of Ro 60-0175 (0.6 mg/kg; Tocris Bioscience, Ellisville, MO) was selected based on previous work showing that it

attenuated effects of nicotine (Fletcher et al., 2012; Higgins et al., 2012) and psychomotor stimulants (Fletcher, Grottick, & Higgins, 2002; Fletcher et al., 2002).

As in Experiments 1 and 2, the effects of an injection of Ro 60-0175 (0.6 mg/kg) or saline on tests of responding for water were assessed. Injections preceded testing by 15 min.

2.4.4 Experiment 4: The effects of M100907 on responding for conditioned reinforcement

Rats were tested 4 times where M100907 (0.5 mg/kg) or vehicle preceded injections of saline or nicotine (0.4 mg/kg) by 30 min. Five min following the second injection, animals were placed in the response chambers for tests of responding for conditioned reinforcement. The dose of M100907 was selected based on reports that this dose occupies >90% of brain 5-HT_{2A} receptors, and blocks other behavioral effects of nicotine and psychomotor stimulants (Fletcher et al., 2002; Fletcher et al., 2012; Knauer et al., 2008). However, the results of this experiment did not show an effect of M100907 to reduce nicotine-enhanced responding for a conditioned reinforcer. To ensure the absence of an effect was not due to an inability of M100907 to override the 0.4 mg/kg dose of nicotine, a separate cohort of animals (n = 10) underwent the same Pavlovian conditioning procedure as the previous experiments, preceded by 0.4 mg/kg nicotine injections. Then, this group was tested 4 times where saline or M100907 (0.5 mg/kg) was administered 30 min prior to vehicle or a lower, 0.2 mg/kg dose of nicotine.

2.5 Drugs

[-]-nicotine bitartrate (Sigma, St. Louis, MO) was dissolved in saline and titrated to a pH of ~7.2. The D_1 receptor antagonist SCH 23390 (0.01 and 0.03 mg/kg; Sigma, St. Louis, MO) and D_2 antagonist eticlopride (0.015 and 0.0075 mg/kg; Sigma, St. Louis, MO) were dissolved in saline and administered SC. The 5-HT_{2C} agonist Ro 60-0175 (0.6 mg/kg; Tocris Bioscience, Ellisville, MO) was dissolved in sterile saline and injected SC. The 5-HT_{2A} receptor antagonist M100907 (0.5 mg/kg; Toronto Research Chemicals) was dissolved in 25 mM acetic acid and 0.3% Tween80 saline solution and pH balanced to ~7.2, and injected SC. All drugs were injected in a volume of 1 ml/kg and doses are expressed in terms of the free base.

2.6 Data Analyses

Data from the tests of responding for the conditioned reinforcer were analyzed using separate three-way ANOVAs for each drug group with Lever (CR/NCR), Nicotine treatment (Saline/Nicotine), and Drug Dose as the independent variables. Violations of sphericity were corrected for using a Greenhouse-Geisser correction for appropriate degrees of freedom. Post-hoc, pairwise comparisons utilized a Tukey's HSD procedure to fix family-wise error rates at $\alpha = 0.05$.

3 Results

3.1 Experiment 1: The Effects of the D_1 Receptor Antagonist SCH 23390 on Nicotine-Enhanced Responding for a Conditioned Reinforcer

In general, animals responded more on the CR versus NCR lever (Figure 1A; main effect of Lever, $F(1, 11) = 43.32$, $p < 0.001$), and nicotine enhanced responding (main effect of Nicotine, $F(1, 11) = 17.17$, $p = 0.002$). The 3-way interaction between Nicotine x Lever x Drug Dose was significant ($F(2, 22) = 12.76$, $p < 0.001$). Further decomposition of the 3-way interaction indicated that nicotine preferentially enhanced responding on the CR lever only (Nicotine x Lever interaction, $F(1, 11) = 20.86$, $p = 0.001$). Both doses of SCH 23390 decreased responding on the CR lever alone, (Main effect of Drug Dose, $F(2, 22) = 92.69$, $p < 0.001$; Lever x Drug Dose interaction, $F(2, 22) = 27.89$, $p < 0.001$) and reduced the ability of nicotine to enhance responding (Nicotine x Drug Dose interaction, $F(2, 22) = 11.06$, $p = 0.002$). Pairwise comparisons indicated that both doses of SCH 23390 prior to nicotine injections significantly ($p < 0.05$) reduced responding on the CR lever compared to levels of responding under the influence of nicotine alone. Both doses of SCH 23390 prior to saline injections also reduced responding compared to saline alone ($p < 0.05$).

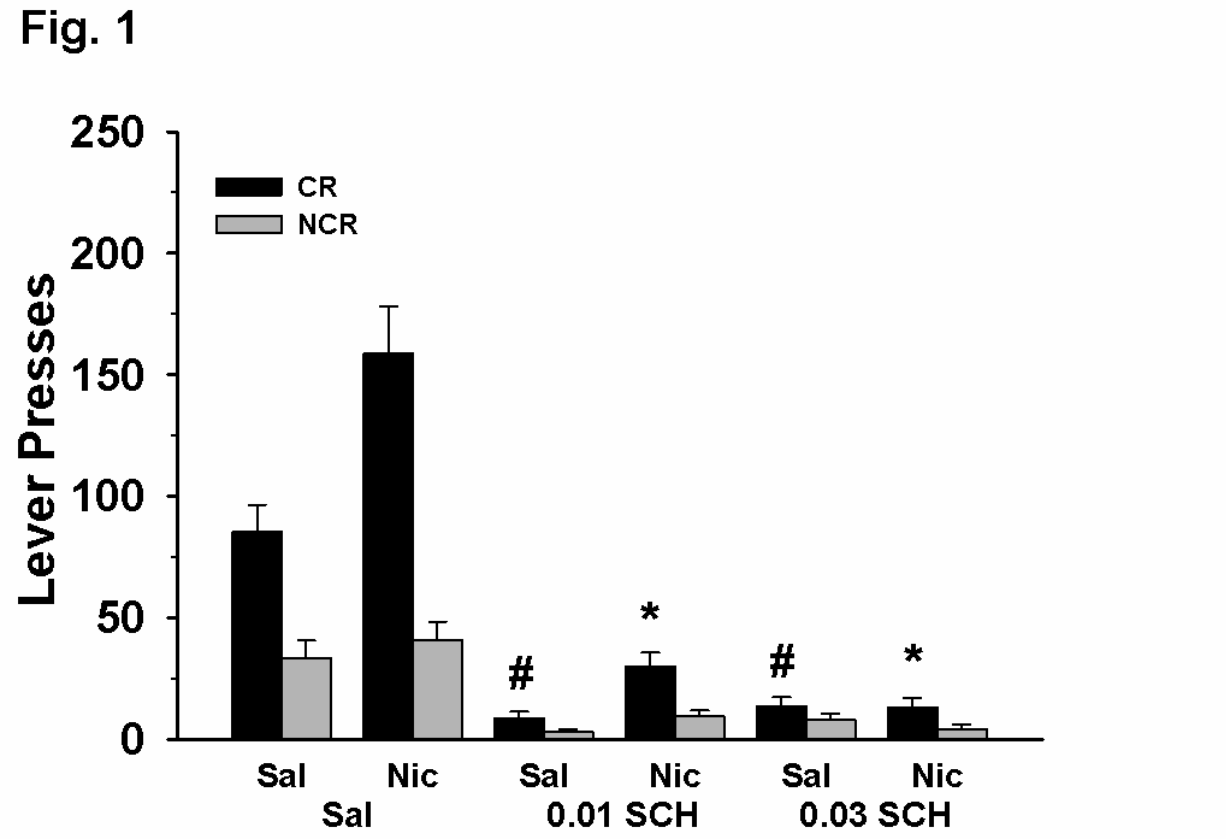

Fig. 1. This figure shows effect of SCH 23390 on nicotine-induced enhancement of responding for a conditioned reinforcer**.** Bars represent the mean (± SEM) number of responses on the reinforced (CR, dark bars) lever and the unreinforced (NCR, grey bars) lever across 6 test days where the effects of nicotine and D_1 receptor antagonism by SCH 23390 were assessed. Test days under the influence of saline or nicotine are indicated on the horizontal axis for each of the following pretreatment conditions (from left to right); saline, 0.01 mg/kg SCH 23390, or 0.03 mg/kg SCH 23390. * indicate an effect of SCH 23390 to significantly ($p < 0.05$) reduce nicotine-enhanced responding on the CR lever. # indicate a reduction ($p < 0.05$) in responding on the CR lever by SCH 23390 compared to the saline test day

3.2 Experiment 1: Responding for a Water Reinforcer

Both doses of SCH 23390 reduced responding for water compared to saline treatment (Figure 5A; $F(2, 22) = 74.45$, $p < 0.001$). However, responding ($M = 236.58$, $SE = 42.26$) at the low

dose of SCH 23390 that also attenuated responding for a conditioned reinforcer was still substantially higher than maximal levels of responding observed during the tests of responding for a conditioned reinforcer (see Figure 1).

3.3 Experiment 2: The Effects of the D_2 Receptor Antagonist Eticlopride on Nicotine-Enhanced Responding for a Conditioned Reinforcer

As shown in Figure 2A, rats preferentially responded on the CR lever (main effect of Lever, $F(1, 11) = 74.49$, $p < 0.001$), and nicotine enhanced responding (main effect of Nicotine, $F(1, 11) = 20.71$, $p = 0.001$). There was a significant 3-way interaction between Nicotine x Lever x Drug Dose ($F(2, 22) = 12.78$, $p < 0.001$). Further analysis of this effect indicated that nicotine preferentially enhanced CR lever responding (Nicotine x Lever interaction, $F(1, 11) = 20.97$, $p = 0.001$). Eticlopride reduced responding on the CR lever (main effect of Drug Dose, $F(2, 22) = 45.17$, $p < 0.001$; Lever x Drug Dose interaction, $F(2, 22) = 21.83$, $p < 0.001$), and the ability of nicotine to enhance this effect (Nicotine x Drug Dose interaction, $F(2, 22) = 19.58$, $p < 0.001$). Post-hoc comparisons indicated that both doses of eticlopride given prior to nicotine significantly (*p-values* < 0.05) lowered responding on the CR lever compared to the test day where the effect of nicotine itself was assessed. In addition, both doses of eticlopride reduced responding on the CR lever compared to saline alone (*p-values* < 0.05).

Fig. 2

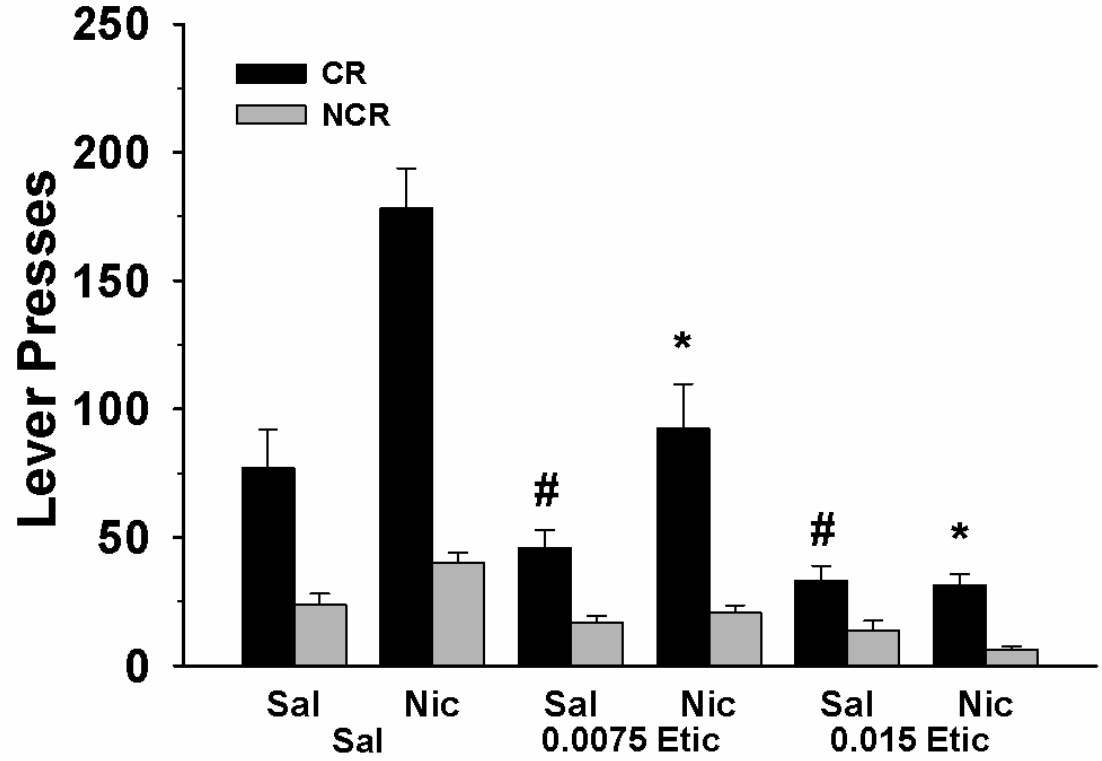

Fig. 2. This figure depicts the effects of two doses of eticlopride on nicotine-enhanced responding for a conditioned reinforcer. Bars depict the mean (± SEM) number of responses on the CR (dark bars) and NCR (grey bars) for the 6 test days conducted to examine the effects of eticlopride and nicotine on responding for a conditioned reinforcer. The horizontal axis labels indicate response levels under the influence of saline or nicotine following pretreatment injections with saline, 0.0075 mg/kg eticlopride, or 0.015 mg/kg eticlopride. * indicate an effect of eticlopride to significantly ($p < 0.05$) reduce nicotine-enhanced responding on the CR lever. # indicate a reduction ($p < 0.05$) in responding on the CR lever by eticlopride compared to the saline test day

3.4 Experiment 2: Responding for a Water Reinforcer

Eticlopride reduced responding for water (Figure 5B; main effect of Drug, $F(2, 22) = 28.76$, $p < 0.001$), but this effect was driven entirely by the highest dose. Post-hoc analyses indicated that the low dose of eticlopride had no effect on overall response rates ($p > 0.05$). Although the high dose reduced responding, ($M = 262$, $SE = 44.57$) this was substantially higher than maximal levels of responding for the conditioned reinforcer (see Fig 2).

3.5 Experiment 3: The Effects of the 5-HT_{2C} Receptor Agonist Ro 60-0175 on Nicotine-Enhanced Responding for a Conditioned Reinforcer

Figure 3A shows responding following nicotine and Ro 60-0175 treatment. Overall, responding was higher on the CR lever compared to the NCR lever (main effect of Lever, $F(1, 9) = 116.92$, $p < 0.001$) and nicotine enhanced response rates (main effect of Nicotine, $F(1, 9) = 21.43$, $p = 0.001$), preferentially on the CR lever (Nicotine x Lever interaction, $F(1, 9) = 56.13$, $p < 0.001$). The 3-way interaction between Nicotine x Lever x Drug Dose did not reach significance ($F(1, 9) = 1.30$, $p > 0.05$). Analysis of the 2-way interactions indicated that the Lever x Drug Dose interaction reached significance ($F(1, 9) = 8.91$, $p = 0.015$), but the Nicotine x Drug Dose interaction did not, ($F(1, 9) = 2.13$, $p > 0.05$). Pairwise comparisons indicated that Ro 60-0175 administration prior to nicotine reduced responding on the CR lever compared to nicotine alone ($p < 0.05$).

3.6 Experiment 3: Responding for a Water Reinforcer

As depicted in Figure 5C, responding for water did not differ between saline or Ro60-0175 test days ($t(9) = 0.152$, $p > 0.05$).

Fig. 3

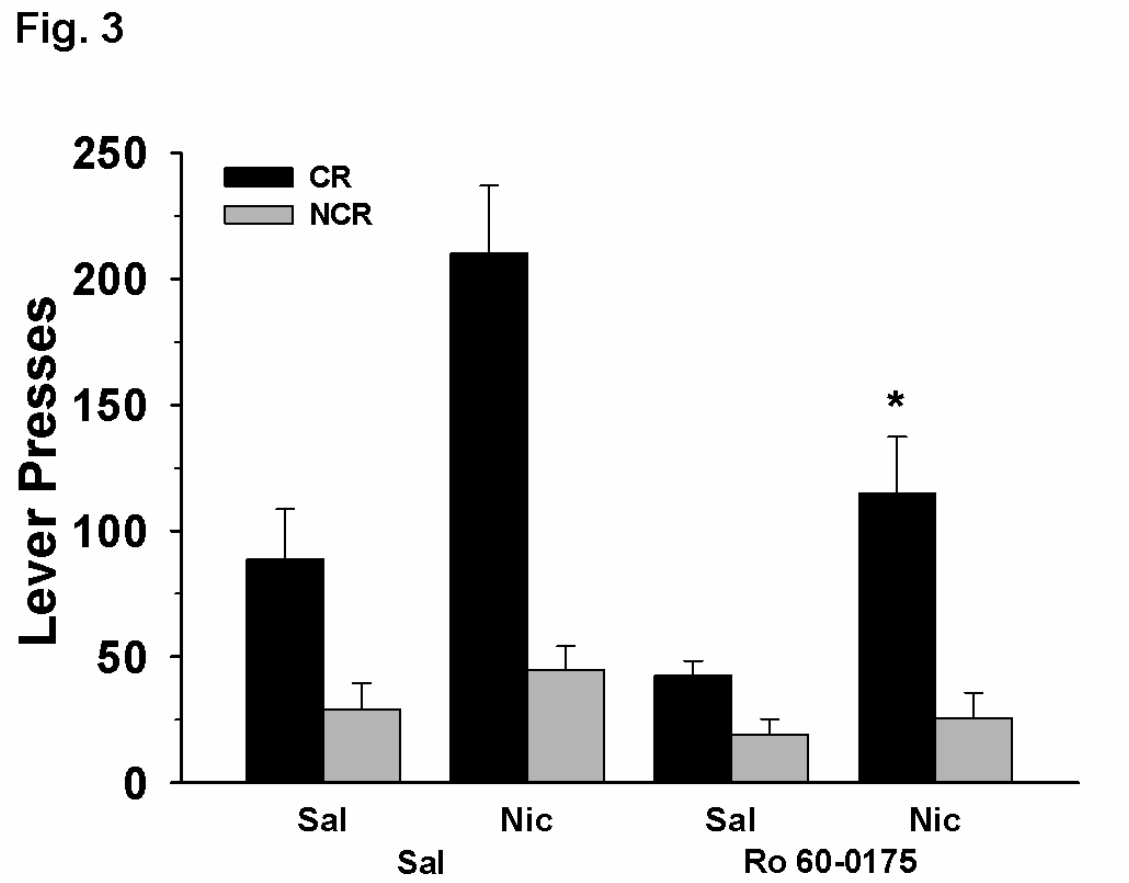

Fig. 3. Depiction of the effect of Ro 60-0175 on nicotine-enhanced responding for a conditioned reinforcer. Bars represent the mean (± SEM) number of lever presses on the CR (dark bars) and NCR (grey bars) levers following injections of saline or nicotine, preceded by a saline injection or administration of the 5-HT_{2C} agonist Ro 60-0175. * indicates an effect of Ro 60-0175 to significantly ($p < 0.05$) reduce nicotine-enhanced responding on the CR lever.

3.7 Experiment 4: The Effects of the 5-HT_{2A} Receptor Antagonist M100907 on Nicotine-Enhanced Responding for a Conditioned Reinforcer

As shown in Figure 4A, animals preferentially responded on the CR lever (main effect of Lever $F(1, 9) = 110.35$, $p < 0.001$), an effect that was enhanced by nicotine injections (main effect of Nicotine, $F(1, 9) = 31.62$, $p < 0.001$; Nicotine x Lever interaction, $F(1, 9) = 22.04$, $p = 0.001$). Injections of M100907 did not significantly affect responding following saline or nicotine injections (*p-values* > 0.05).

3.8 Experiment 4: The effects of 5-HT_{2A} receptor antagonism by M100907 on nicotine-enhanced responding for a conditioned reinforcer with 0.2 mg/kg nicotine

As displayed in Figure 4B, rats preferentially responded on the CR lever (main effect of Lever $F(1, 9) = 34.95$, $p < 0.001$). The 0.2 mg/kg dose of nicotine enhanced this preferential responding (main effect of Nicotine, $F(1, 9) = 54.28$, $p < 0.001$; Nicotine x Lever interaction, $F(1, 9) = 29.14$, $p < 0.001$). Again, there was no effect of M100907 to alter these response patterns (*p-values* > 0.05).

Fig. 4

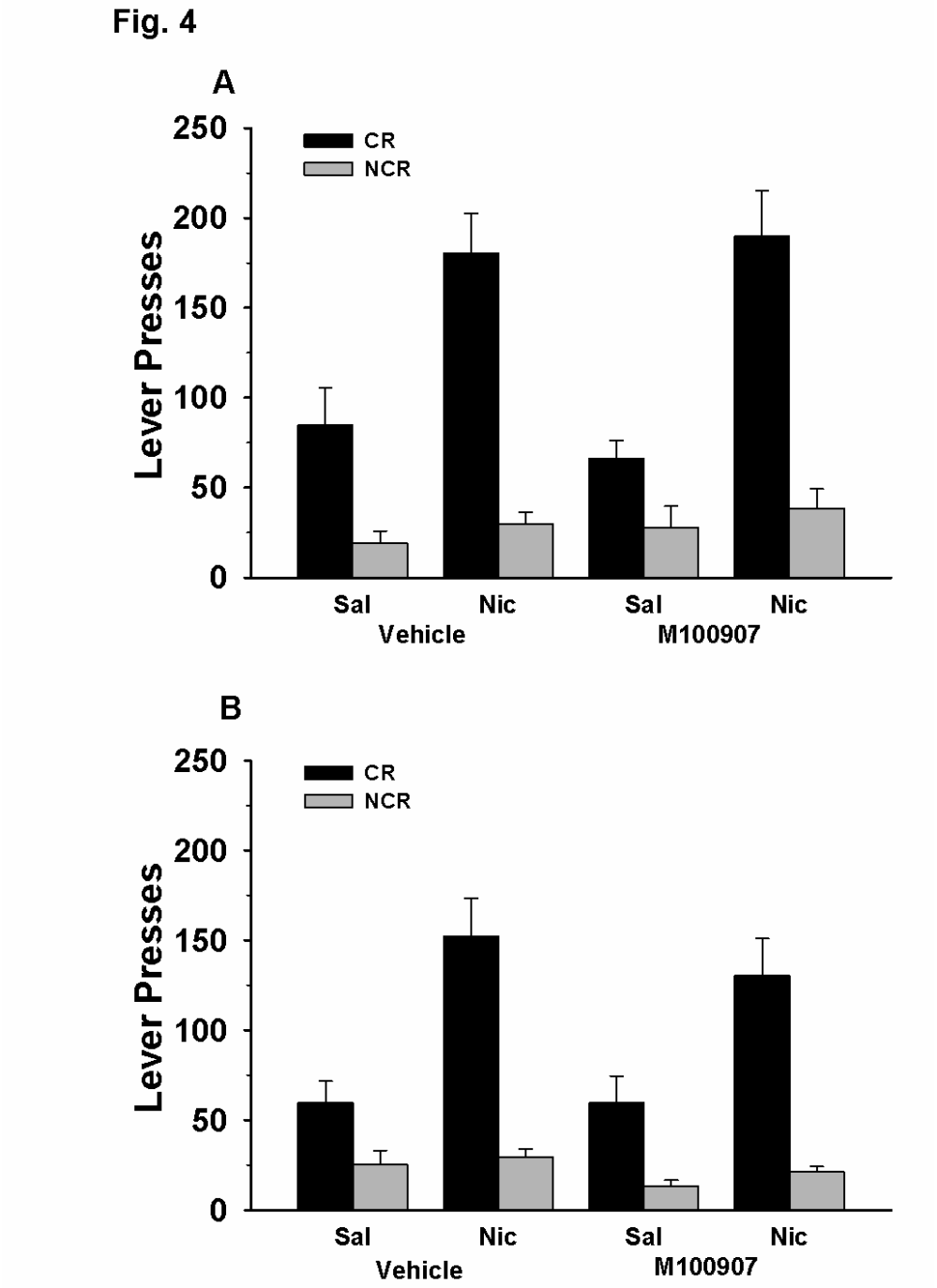

Fig. 4. This figure displays the effects of M100907 on the ability of a 0.4 mg/kg (Panel A) or 0.2 mg/kg (Panel B) dose of nicotine to enhance responding for a conditioned reinforcer. Bars depict the mean (± SEM) number of lever presses on the CR (dark bars) and NCR (grey bars) levers following injections of saline or nicotine, preceded by the administration of the vehicle or injections of the 5-HT_{2A} antagonist M100907.

Fig. 5

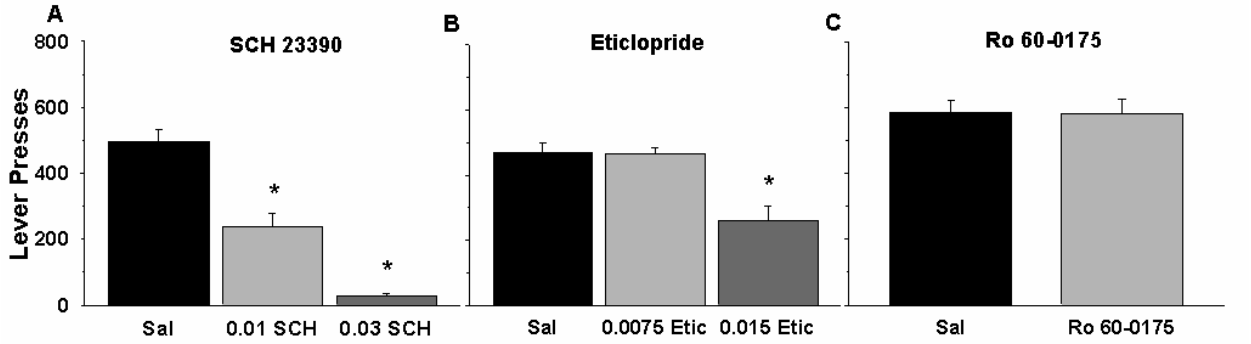

Fig. 5. The effects of SCH 23390, eticlopride, or Ro 60-0175 on responding for water in the absence of any CSs. Panel A shows the average (± SEM) level of lever pressing for the delivery of water reinforcement under saline, 0.01 or 0.03 mg/kg of SCH 23390. Panel B displays the average number of responses for a water reinforcer under saline and the 0.015 and 0.0075 mg/kg doses of eticlopride. Panel C depicts responding for water under saline or the 0.6 mg/kg dose of Ro 60-0175. * signifies an effect of SCH 23390 or eticlopride to reduce responding ($p < 0.05$) for the water reinforcer

4 Discussion

These experiments found that drugs targeting DA and 5-HT receptors affect nicotine-enhanced responding for conditioned reinforcement. The preferential D_1 receptor antagonist SCH 23390 and the D_2 receptor antagonist eticlopride decreased responding for conditioned reinforcement, and attenuated the ability of an acute injection of nicotine to enhance this response. Likewise, the preferential 5-HT_{2C} receptor agonist Ro 60-0175 attenuated responding for conditioned reinforcement, and blocked the effect of nicotine to enhance this behavior. The 5-HT_{2A} receptor antagonist M100907 did not affect responding for the conditioned reinforcer, or the enhancement of this response by nicotine. Together, these findings expand upon prior research demonstrating that nicotinic receptor activation enhances the motivational properties of conditioned reinforcers (Brunzell et al., 2006; Guy & Fletcher, 2012; Löf, Olausson, Stomberg, Taylor, & Söderpalm, 2010; Olausson et al., 2004a; Olausson, Jentsch, & Taylor, 2004b) by implicating dopamine and serotonin as additional neurotransmitter systems that may interact with the ability of nicotine to affect this behavioral measure of incentive motivation.

In keeping with the design of numerous experiments examining the effects of a variety of drugs, or drug combinations, on responding for a conditioned reinforcer (Fletcher, 1996; Kelley & Delfs, 1991; Taylor & Robbins, 1984; Wolterink et al., 1993), we tested all drug doses in combination with saline or nicotine using a repeated measures design. Our previous work has shown that responding for a conditioned reinforcer is maintained over multiple test trials (Guy & Fletcher, 2013a). More importantly, for the purposes of this study, nicotine-induced enhancement of responding for conditioned reinforcement was found to be stable after multiple tests (Guy & Fletcher, 2013a), and even re-established responding for the conditioned reinforcer after the response had been extinguished (Guy & Fletcher, 2013b). Thus, the effects of these drugs to reduce responding for conditioned reinforcement, and the ability of nicotine to enhance this response, likely reflect the acute behavioral effects of the drug rather than factors related to alterations in the reinforcing properties of the conditioned reinforcer arising from repeated testing.

The finding that blockade of either DA D_1 or D_2 receptors decreased responding for a conditioned reinforcer, and the effect of nicotine to enhance this measure, is consistent with the substantial role for dopamine in enhancing reward-seeking behaviors in response to the presence

of reward-predictive CSs (Berridge & Robinson, 1998; Robinson & Berridge, 1993). In tests of responding for a conditioned reinforcer, as used in the present study, numerous reports have identified a fundamental role for the mesolimbic dopaminergic system. Drugs that increase dopaminergic activity, such as amphetamine or pipradrol, enhance responding for conditioned reinforcement (Beninger et al., 1980; Robbins, 1978; Taylor & Robbins, 1984; Taylor & Robbins, 1986; Wolterink et al., 1993). Additionally, microinjections of dopamine agonists into the NAc enhance responding for conditioned reinforcers (Kelley & Delfs, 1991; Taylor & Robbins, 1984; Wolterink et al., 1993). This effect can be blocked by systemic dopamine antagonists (Ranaldi & Beninger, 1993), microinfusions of dopamine antagonists directly into the NAc (Wolterink et al., 1993), or by depletion of NAc DA (Taylor & Robbins, 1986), suggesting that dopamine receptor activation, particularly within the NAc is critical to the response-potentiating effects of psychostimulants. In relation to nicotine-reinforced behaviors, manipulations that reduce dopaminergic activity, such as selective lesions of mesolimbic DA neurons with 6-hydroxydopamine (6-OHDA; Corrigall et al., 1992) or systemic antagonism of D_1 or D_2-like dopamine receptors (Corrigall & Coen, 1991), dramatically reduce responding for the combination of a CS and intravenous nicotine. Following extinction of nicotine self-administration, the reactivation of operant behavior on the lever previously paired with nicotine reinforcement by a nicotine-paired CS is also blunted by systemic D_1 or D_2 receptor antagonism (Liu et al., 2011). Thus, our data complement existing evidence that DA receptor stimulation is involved in enhancing conditioned reinforcement in general, and indicate that DA receptor tone is necessary for any response-potentiating effects of nicotine to manifest.

The possibility that the reductions in responding for conditioned reinforcement by pharmacological blockade of D_1 or D_2 receptors was due to a compromised ability to respond on the levers was assessed in follow up studies where rats responded for water under the influence of both doses of SCH 23390 or eticlopride. Here, response rates were much higher than when rats were responding for a conditioned reinforcer. The highest dose of the D_1 receptor antagonist SCH 23390 induced a large reduction in responding for water, indicating some possible drug-induced performance effects. The low dose of SCH 23390 also reduced responding compared to saline, but response levels were still above the highest observed levels maintained by the conditioned reinforcer. The high dose of eticlopride also attenuated responding for water, but again response rates were still above those observed in tests of responding for conditioned

reinforcement. The low dose of eticlopride had no effect on this behavior. In tests of self-administration of cocaine (Hubner & Moreton, 1991; Roberts, Loh, & Vickers, 1989) or methylphenidate (Botly et al., 2008), SCH23390 actually *enhanced* rates of responding for these drugs. The same dose of eticlopride used in this study has also been shown to enhance responding for methylphenidate (Botly et al., 2008). Therefore, an effect on incentive motivational processes, rather than an impaired ability to respond on the lever, is more likely to have contributed to the effect of eticlopride to reduce responding for conditioned reinforcement, and interfere with the ability of nicotine to enhance this response.

Our finding that the 5-HT_{2C} receptor agonist Ro 60-0175 reduces responding for conditioned reinforcement is in accord with the proposed role for these receptors in reducing incentive motivation, presumably via tonic inhibitory control over mesolimbic DA release (Higgins & Fletcher, 2003). The result of this effect is a reduction in the dopaminergic tone likely needed for nicotine administration to induce any response-potentiating effects for conditioned reinforcement. Other reports support a similar role for 5-HT_{2C} receptor activation in reducing operant behaviors reinforced by CSs. For example, microinjections of 5-HT in the NAc diminish the facilitation of responding for a conditioned reinforcer by amphetamine (Fletcher, 1996). The 5-HT releaser MDMA also reduces responding for conditioned reinforcement, and the blockade of 5-HT_{2C} receptors by SB242084 reverses the suppressant effect of MDMA on this response, suggesting 5-HT_{2C} receptor stimulation mediates the effect of 5-HT on reducing responding for conditioned reinforcement (Fletcher et al., 2002). 5-HT_{2C} receptors also affect the motivating properties of drug-paired CSs, specifically the ability of these CSs to reinstate extinguished drug-seeking behaviors. In animals with a history of cocaine and nicotine self-administration, systemic injections of 5-HT_{2C} receptor agonists block the reinstatement of extinguished responding on the previously drug-paired lever by both the drug prime and the CS previously paired with intravenous drug delivery, and these effects are reversed by the 5-HT_{2C} antagonist SB242084 (Fletcher et al., 2012; Higgins et al., 2012; Neisewander & Acosta, 2004). Together, our findings and these reports indicate that 5-HT_{2C} receptor activation can reduce motivated behaviors reinforced by CSs, and the enhancement of these behaviors by nicotine administration.

The possible effect of Ro 60-0175 to reduce the ability to respond on the lever was also assessed in a test of responding for the primary reinforcer alone. Response levels in this measure did not differ from saline, ruling out this possible effect on motor behavior in the observed results in the

test of responding for a conditioned reinforcer. The lack of effect of Ro 60-0175 on responding for water also suggests that primary reinforcement processes remained intact, and that the effect of Ro 60-0175 to reduce responding for conditioned reinforcement was due to a specific decrease in the motivating properties of the conditioned reinforcer.

In contrast to the 5-HT_{2C} receptor subtype, the stimulation of 5-HT_{2A} receptors facilitates mesolimbic dopaminergic responses induced by psychostimulant administration (Higgins & Fletcher, 2003; Di Matteo, Giovanni, Mascio, & Esposito, 1999; Zaniewska, McCreary, & Filip, 2009). Considering the role for mesolimbic DA in responding for conditioned reinforcement, it was hypothesized that the blockade of 5-HT_{2A} receptors would reduce nicotine-induced enhancements in responding for conditioned reinforcement. Behaviorally, the systemic blockade of 5-HT_{2A} receptors by M100907 exerts a similar influence to Ro 60-0175 on blunting the reinstatement of extinguished drug-seeking behavior by the drug-associated CS or a drug prime (Fletcher et al., 2012; Fletcher, et al., 2002). However, in the current experiments, the effects of M100907 contrasted with that of Ro 60-0175. The systemic blockade of 5-HT_{2A} receptors did not alter responding for conditioned reinforcement, nor did it alter the ability of 0.4 mg/kg nicotine to increase this behavior.

The dose of 0.5 mg/kg M100907 used in this study produces a functional blockade of 5-HT_{2A} receptors (Kehne, et al., 1996), and reduces the effects of psychostimulant drugs to activate locomotor behaviors (Kehne, et al., 1996; McMahon & Cunningham, 2001). However, it is possible that even this level of 5-HT_{2A} receptor blockade may not be able to overcome the effects of a high dose of nicotine on reinforced behaviors. To test this possibility, a second experiment combined M100907 with 0.02 mg/kg nicotine. This dose of nicotine enhanced responding for the conditioned reinforcer, but to a lower degree than did 0.4 mg/kg. Again, M100907 did not affect this response.

The lack of effect of M100907 on responding for a conditioned reinforcer in these two experiments contrasts with its effects to reduce cue or nicotine induced reinstatement of extinguished drug seeking behavior (Fletcher et al., 2012). One reason for these seemingly discrepant effects of M100907 may relate to differences in the neural mechanisms underlying cue-evoked reinstatement of drug-seeking behavior as compared to responding for a conditioned reinforcer. In particular, manipulations within the prefrontal cortex (PFC) have suggested

differential involvement of this brain region for these two cue-motivated behaviors. For example, microinjections of M100907 into the PFC reduces the reinstatement of cocaine-seeking behavior by a cocaine-paired CS (Pockros, Pentkowski, Swinford, & Neisewander, 2011). However, lesion studies have shown that the PFC does not appear to mediate the ability of a CS paired with natural reinforcement to support the acquisition of a new response as a conditioned reinforcer (Burns, Robbins, & Everitt, 1993). It has been proposed that the effect of 5-HT_{2A} receptor stimulation to enhance mesolimbic DA release depends on the precise neuronal ensembles activated. For example, the activation of 5-HT_{2A} receptors enhances amphetamine-induced increases in mesolimbic DA release, but not morphine-induced increases in mesolimbic DA activity (Porras et al., 1999). While the PFC does send projections to the NAc (Everitt & Robbins, 2005), the specific population of neurons activated within the accumbens during cue-evoked reinstatement of self-administration behavior may differ from those activated during tests of responding for a conditioned reinforcer, with the former being less sensitive to PFC modulation of dopaminergic projections by 5-HT_{2A} receptor antagonism.

4.1 Concluding Remarks

The results from these experiments are consistent with evidence that nicotine enhances the reinforcing properties of reward-related stimuli. In addition, these data further characterize some of the neurochemical mechanisms underlying this effect of nicotine. The identification of roles for both D_1 and D_2 receptors in the response-potentiating effect of nicotine on responding for a conditioned reinforcer support the substantial evidence implicating dopaminergic neurotransmission in the reinforcing properties of nicotine and nicotine-associated CSs (Balfour et al., 2002; Corrigall, Coen, & Adamson, 1994; Corrigall & Coen, 1991; Corrigall et al., 1992; Liu et al., 2011). Furthermore, the results from Experiment 3 indicated that manipulations of 5-HT_{2C} receptor activation can modify CS-motivated behaviors and the impact of nicotine on responding for these cues. This supports emerging evidence supporting the use of 5-HT_{2C} receptor agonists to reduce nicotine self-administration and the reinstatement of nicotine-seeking behaviors (Fletcher et al., 2012). In addition, the present results contribute to the evidence in favor of targeting this receptor subtype in therapeutic interventions for smoking abstinence (Higgins, Sellers, & Fletcher, 2013; Higgins et al., 2012; Higgins et al., 2012). Together, these data indicate that nicotine-enhanced responding for a conditioned reinforcer can be used to identify the some of the neural mechanisms by which nicotine invigorates the motivating

properties of reward-associated stimuli, which likely contribute to the development and maintenance of tobacco smoking.

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Chapter 6
Examination of the Effects of Varenicline, Bupropion, Lorcaserin, or Naltrexone on Responding for Conditioned Reinforcement in Nicotine-Exposed Rats

Abstract

Smoking tobacco remains one of the leading causes of preventable deaths in North America. Nicotine reinforces smoking behavior, in part, by enhancing the reinforcing properties of reward-related stimuli, or conditioned stimuli (CSs), associated with tobacco intake. To investigate how pharmaceutical interventions may affect this property of nicotine, we examined the effect of four Federal Drug Administration (FDA)-approved pharmaceuticals on the ability of nicotine to enhance operant responding for a CS as a conditioned reinforcer. Thirsty rats were exposed to 13 Pavlovian sessions where a CS was paired with water delivery. Nicotine (0.4 mg/kg) injections were administered prior to each session. Then, in separate groups of rats, the effects of varenicline (1 mg/kg), bupropion (10 and 30 mg/kg), lorcaserin (0.6 mg/kg), and naltrexone (2 mg/kg) and their interaction with nicotine on responding for conditioned reinforcement were examined. Varenicline and lorcaserin both blocked nicotine-enhanced responding for conditioned reinforcement, bupropion enhanced responding, and naltrexone had no effect on responding for conditioned reinforcement, but modestly reduced responding enhanced by nicotine to a level that did not differ from the saline condition. The results of these studies may inform how pharmaceutical interventions can affect smoking cessation attempts and relapse through diverse mechanisms; either substituting for, or interacting with, the reinforcement-enhancing properties of nicotine.

1 Introduction

Current estimates indicate that approximately 19.3% of all adults in the United States smoke cigarettes on a regular basis (CDC, 2013). Tobacco use is associated with adverse health outcomes, such as coronary heart disease, chronic obstructive pulmonary disease (COPD), and cancer; making smoking one of the leading causes of preventable deaths in North America (CDC, 2013; George & O'Malley, 2004; Polosa & Benowitz, 2011). Nicotine is the primary psychoactive ingredient in tobacco smoke that reinforces smoking (Stolerman & Jarvis, 1985). Pharmaceutical interventions, such as nicotine replacement therapy, varenicline and bupropion, have shown some efficacy in abating tobacco use, but quit rates still remain low, hovering around the 20% range (George & O'Malley, 2004; Polosa & Benowitz, 2011). Furthermore, these interventions can have unwanted side-effects (e.g., insomnia, heart arrhythmias) and contraindications with other pharmaceuticals (Polosa & Benowitz, 2011). A more comprehensive understanding of the neural mechanisms by which these drugs have their therapeutic effects is needed in order to improve abstinence rates, and such information could aid in the development of more tailored interventions with fewer unwanted side-effects.

Nicotine use is perpetuated, in part, by an effect to enhance the reinforcing properties of reward-associated cues, or conditioned stimuli (CSs). These CSs may be either directly paired with nicotine intake (e.g., smoke and taste of tobacco), or in close proximity (e.g., alcohol, social interactions; Caggiula et al., 2001). Numerous reports in animal and human research indicate that CSs paired with nicotine administration are at least as important as the nicotine dose itself in reinforcing nicotine intake (Balfour, Wright, Benwell, & Birrell, 2000; Caggiula et al., 2001; Chiamulera, 2005; Rose, Behm, Westman, & Johnson, 2000), and reducing the reinforcing properties of these cues may help curb tobacco consumption (Caggiula et al., 2001).

The motivating properties of nicotine-associated CSs have been examined using several different techniques. In self-administration studies, the presentation of a CS previously paired with nicotine infusions reactivates extinguished nicotine-seeking behavior (Higgins et al., 2012; LeSage, Burroughs, Dufek, Keyler, & Pentel, 2004; Liu et al., 2006; Shaham, Adamson, Grocki, & Corrigall, 1997). This phenomenon may reflect in part the ability of the CS to serve as a conditioned reinforcer. Another, more definitive test of the acquired motivational properties of a reward-associated CS is the ability of that stimulus to support a novel operant response that has

never been associated with any reinforcement (Everitt & Robbins, 2005; Saunders & Robinson, 2013). The degree of operant responding for the CS reflects its motivational properties (Mackintosh, 1974), and nicotine enhances this response (Guy & Fletcher, 2013; Olausson, Jentch, & Taylor, 2004). Thus, responding for conditioned reinforcement, and its potentiation by nicotine, may be a useful measure of the motivational properties of CSs in nicotine reinforcement, and a method to examine the impact of pharmaceutical interventions on this effect.

Available pharmaceuticals that may be used for smoking cessation include drugs that target nicotinic acetylcholine receptors (nAChRs); or modify dopamine (DA) activity, which has been known to affect the motivating properties of reward-associated cues. Possible novel interventions may interact with other neurotransmitter systems besides DA that alter cue-elicited motivation, such as serotonin (5-HT) or opioid systems (Corrigall & Coen 1991; Corrigall, Franklin, Coen, & Clarke, 1992; Higgins et al., 2012; Levin et al., 2011; Liu et al., 2009). The *β2-containing nAChRs have been heavily implicated in various forms of nicotine reinforcement (Picciotto et al., 1998; Rollema et al., 2007; Tobey et al., 2012) and the reactivation of nicotine-seeking behaviors in rodents and humans after periods of extinguished responding. Reflecting this role, the α4β2 partial agonist and α7 nAChR receptor full agonist varenicline blocks both nicotine and nicotine paired with cue-induced reinstatement of self-administration behavior in rats (Mihalak, Caroll, & Luetje, 2006; O'Connor, Parker, Rollema, & Mead, 2010). Similarly, varenicline reduces subjective cravings and associated neuronal activity among human smokers exposed to smoking cues (Franklin et al., 2011). Clinical trials have indicated that the DA and NE reuptake inhibitor bupropion improves quit rates compared to placebo (Warner & Shoaib, 2005), and some report that it reduces nicotine self-administration behavior in rats (Glick, Maisonneuve, & Kitchen, 2002; Rauhut, Dwoskin, & Bardo, 2005). However, this drug has not demonstrated an effect of reducing cue-associated cravings in humans, or the motivating properties of reinforcing stimuli in rats (Palmatier et al., 2009a; Shoaib, Sidhpura, & Shafait, 2003). Drugs that target 5-HT receptors, particularly the 5-HT_{2C} receptor agonists Ro 60-0175 and lorcaserin, can also block cue-evoked nicotine reinstatement (Fletcher et al., 2012; Higgins et al., 2012) and reduce responding for a conditioned reinforcer (Fletcher, Korth, Robinson, & Baker, 2002). Within the opiate system, the blockade of *mu*-opioid receptors by naltrexone has been shown to inhibit the cue-evoked reinstatement of nicotine-seeking behavior in rats (Liu et

al., 2009). However, the ability of such antagonists to curb cigarette smoking in humans remains equivocal (David, Lancaster, Stead, & Evins, 2009).

The following studies were designed to examine how drugs with distinct pharmacological effects interact with the reinforcing properties of reward-associated CSs, and their modulation by nicotine. Specifically, separate experiments examined the effect of administering the α4β2 partial agonist varenicline (Mihalak et al., 2006; Rollema et al., 2007), the DA and NE reuptake inhibitor bupropion (George & O'Malley, 2004), the 5-HT_{2C} receptor agonist lorcaserin (Higgins et al., 2012; Levin et al., 2011), and the μ-opioid receptor antagonist naltrexone (Liu et al., 2009) on nicotine-induced enhancements in responding for conditioned reinforcement.

2 Methods

2.1 Subjects

Fifty-one male Long-Evans rats (Charles River, Quebec, Canada) weighing 225-250g upon arrival were singly housed in a temperature (~22°C) and humidity-controlled (~50-60%) vivarium on a 12 hour light/dark cycle (lights on 0700 h-off 1900 h). Food was available at all times, but water access was restricted as described below. All procedures were approved by the Centre for Addiction and Mental Health (CAMH) Animal Care Committee and adhered to Canadian Tricouncil guidelines for the humane treatment of experimental animals.

2.2 Drugs

All doses are expressed as the base amount of drug. [-]-nicotine bitartrate (Sigma, St. Louis, MO) was dissolved in sterile 0.9% saline and titrated to a pH of ~7.2 and injected (0.4 mg/kg, SC) 5 min prior to behavioral tests. A 1 mg/kg dose of the α4β2 partial agonist varenicline tartrate (Toronto Research Chemicals, Toronto, Canada) was selected because this dose blocks the reinstatement of self-administration by nicotine and a CS, and it antagonizes nicotine's effect of enhancing responding for other, non-drug stimuli (Levin et al., 2012; O'Conner et al., 2010). We examined the effects of two doses of the DA and NE reuptake inhibitor bupropion HCl (10 mg/kg and 30 mg/kg; Toronto Research Chemicals, Toronto, ON) based on evidence that doses within this range may have different effects to increase or decrease responding for nicotine and other reinforcers (Palmatier et al., 2009; Rauhut et al., 2003; Shoaib et al., 2003). The 0.6 mg/kg dose of the 5-HT_{2C} receptor agonist lorcaserin HCl (NPS Pharmaceuticals, Toronto, Canada)

blocks both nicotine self-administration and reinstatement (Higgins et al., 2012, 2013), and was selected based on this evidence. Finally, we tested 2mg/kg of the μ-opioid antagonist naltrexone HCl (Toronto Research Chemicals, Toronto, ON) because this dose blocks reinstatement of nicotine-seeking behavior by a CS and nicotine prime. All drugs were dissolved in 0.9% saline and injected prior to nicotine, as described below.

2.3 Pavlovian Conditioning

All training and testing occurred in operant conditioning chambers (Med Associates, St. Albans, VT, USA) containing two retractable levers located 6.5 cm either side of a recessed water delivery receptacle positioned 3 cm from the floor of the chamber. An infrared photocell detector within the receptacle recorded head entries. A stimulus light was located above each response lever. Chambers were housed within sound-attenuating cubicles. The day prior to the first Pavlovian conditioning session, animals were restricted to 1 h of free water access and remained restricted to 1 h of access following each experimental session throughout the conditioning and testing procedures. Each ~30 min Pavlovian conditioning session consisted of 30 pairings of a 5 s CS followed immediately by the presentation of 0.05 mL of tap water (US), delivered by a solenoid operated water dispenser on a random time (RT) 60 s schedule of reinforcement. The CS consisted of a 5 s illumination of the two red stimulus lights with the houselights turned off and a 2.9 kHz, 85 dB tone stimulus (Sonalert) presented during the last 0.5 s of the light presentation. All animals were administered nicotine (0.4 mg/kg, SC) injections just prior to each Pavlovian session. Prior reports indicate this dosing regimen results in enhanced responding for conditioned reinforcement when rats are subsequently challenged with nicotine (Guy & Fletcher, 2013).

2.4 Responding for Conditioned Reinforcement

During tests of responding for conditioned reinforcement, two levers were inserted into the chambers. Responding on one lever resulted in presentation of the CS, in the absence of the water reward, on a RR2 schedule of reinforcement (i.e., each press on the reinforced lever had a 0.5 probability of reinforcement). This lever was designated the conditioned reinforcer (CR) lever. Responses on the other lever, designated the NCR lever, had no programmed consequences. CR and NCR lever responses were recorded. Animals were randomly assigned to one of five groups where the effects of varenicline (1 mg/kg, SC, n = 9), two doses of

bupropion (10 mg/kg or 30 mg/kg, IP, n = 10 per group), lorcaserin (0.6 mg/kg, SC, n = 10), and naltrexone (2 mg/kg, SC, n = 12) were separately tested. Using a within-subjects design, each rat was tested under the four possible combinations of the pharmaceutical pretreatment or vehicle and nicotine or saline, randomized across subjects with a Latin squares design. Drug test days were separated by a minimum of 72 h. Naltrexone, varenicline, and bupropion were administered 30 min prior to nicotine and lorcaserin was administered 10 min prior to nicotine.

2.5 Responding for Water

Lorcaserin was the only drug to lower responding for conditioned reinforcement when administered alone. To test for possible effects on responding for the primary reinforcer, rats from the conditioned reinforcement study were trained in 30 m sessions to respond on the lever previously unpaired with conditioned reinforcement for 0.05 mL delivery of water on a random-ratio (RR2) schedule of reinforcement. Once responding stabilized (i.e., response levels were consistent for at least 2 consecutive sessions) two test sessions were conducted, preceded by saline or lorcaserin (0.6 mg/kg, SC) injections. Test days were separated by 72 h, and drug order was randomized.

2.6 Statistical Analyses

Statistical analyses were conducted using SPSS version 15.0. Data from tests of responding for conditioned reinforcement were analyzed using separate, three-way ANOVAs for each drug group (Varenicline/ 10 mg/kg Bupropion/ 30 mg/kg Bupropion/ Lorcaserin/ Naltrexone). Lever (active/inactive lever), Nicotine treatment (saline/nicotine), and Drug treatment (saline/drug treatment) were the independent variables. Violations of sphericity were corrected for using a Greenhouse–Geisser correction for appropriate degrees of freedom. Post-hoc pairwise comparisons utilized a Tukey's HSD procedure to fix family-wise error rates at $\alpha < 0.05$.

3 Results

3.1 Varenicline

As shown in Figure 1, responding on the CR lever was greater than on the unreinforced, NCR lever (main effect of Lever $F(1, 7) = 46.41$, $p < 0.001$). Nicotine did not affect this pattern of preferential responding on the CR lever (Nicotine x Lever interaction, $F(1, 7) = 1.87$, $p = 0.214$),

but nicotine did enhance responding overall (main effect of Nicotine, $F(1, 7) = 7.54$, $p < 0.029$). Varenicline blocked the ability of nicotine to enhance lever pressing behavior (Lever x Varenicline x Nicotine interaction, $F(1, 7) = 38.35$, $p < 0.001$; Varenicline x Nicotine interaction, $F(1, 7) = 31.49$, $p = 0.001$). Further pairwise comparisons indicated that varenicline itself significantly enhanced responding on the CR lever compared to saline ($p < 0.05$), but varenicline co-administered with nicotine blocked any potentiation of responding for CR ($p > 0.05$ compared to saline).

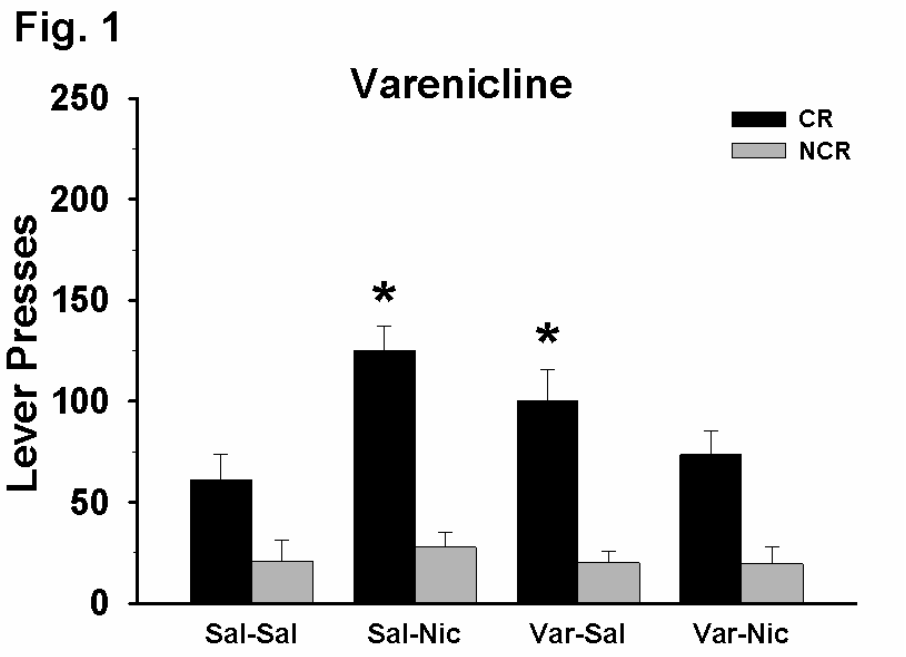

Fig. 1. The effect of varenicline (Var) on nicotine-enhanced responding for a conditioned reinforcer. Bars depict the average (± SEM) response levels on the lever paired with the conditioned reinforcer (CR lever, dark bars) and the lever with no programmed consequences (NCR lever, grey bars) for the four different test days, indicated on the horizontal axis. Descriptions before the dash indicate whether saline or varenicline was administered prior to injections of saline or nicotine just prior to testing (indicated after the dash). * indicates a significant enhancement of responding on the CR lever compared to the Sal-Sal test condition ($p < 0.05$).

3.2 Bupropion

As depicted in Figure 2, both the 10 mg/kg and 30 mg/kg bupropion groups showed a preference for responding on the CR lever compared to the NCR lever (main effect of Lever, $F(1,9) = 54.45$, $p < 0.001$; and $F(1,9) = 56.02$, $p < 0.001$ for 10 mg/kg and 30 mg/kg, respectively). Nicotine enhanced this effect in both groups (main effect of Nicotine for 10 mg/kg group, $F(1, 9) = 21.28$, $p = 0.001$, Nicotine x Lever interaction, $F(1, 9) = 21.60$, $p = 0.001$; main effect of Nicotine for the 30 mg/kg group, $F(1, 9) = 14.68$, $p = 0.004$, Nicotine x Lever interaction, $F(1, 9) = 9.52$, $p = 0.013$). The 10 mg/kg dose of bupropion did not affect responding for conditioned reinforcement or the ability of nicotine to enhance this effect (main effect of Bupropion, $F(1, 9) = 0.11$, $p > 0.05$; Bupropion x Nicotine interaction, $F(1, 9) = 0.098$, $p > 0.05$). In contrast, the 30 mg/kg dose of bupropion increased responding for a conditioned reinforcer in both the drug free and nicotine tests (main effect of Bupropion, $F(1, 9) = 15.40$, $p = 0.003$). Post-hoc comparisons indicated that 30 mg/kg bupropion alone enhanced responding for conditioned reinforcement to the same level as nicotine alone, and when combined with nicotine enhanced responding on the CR lever to a level above that for nicotine or bupropion alone (*p- value* < 0.05).

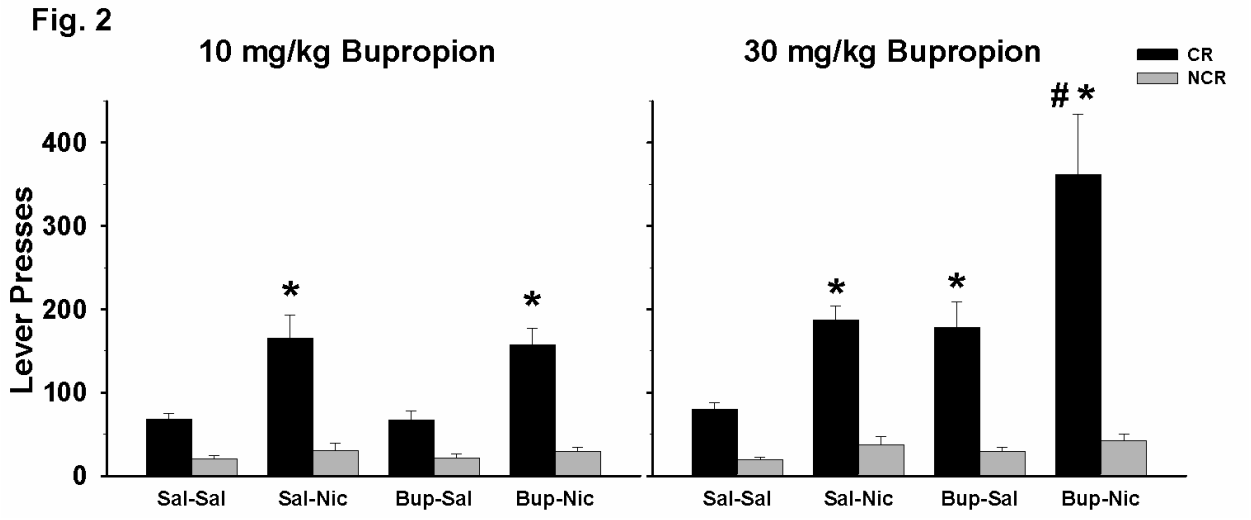

Fig. 2. The effects of two doses of bupropion (Bup) on nicotine-enhanced responding for conditioned reinforcement. Mean (± SEM) levels of responding on the CR (dark bars) compared to the NCR (grey bars) are represented. Panel A displays the effect of saline (Sal) or a low (10 mg/kg) dose of bupropion administered prior to saline (Sal) or nicotine (Nic) on operant behavior, as indicated on the horizontal axis. Panel B shows the effects of a high (30 mg/kg) dose of bupropion on such responding. * demarks an enhancement in responding on the CR lever compared to the Sal-Sal condition ($p < 0.05$). # indicates enhanced responding on the CR lever compared to the Sal-Nic condition ($p < 0.05$).

3.3 Lorcaserin

Figure 3A shows that rats demonstrated preferential responding for the CR lever compared to the NCR lever (main effect of Lever $F(1, 9) = 24.98$, $p = 0.001$) and nicotine enhanced this effect (main effect of Nicotine, $F(1, 9) = 22.36$, $p = 0.001$; Nicotine x Lever interaction, $F(1,9) = 16.02$, $p = 0.003$). Responding for conditioned reinforcement in general, and the effect of nicotine to enhance responding for the conditioned reinforcer, was decreased by lorcaserin pretreatments (main effect of Lorcaserin, $F(1,9) = 47.41$, $p < 0.001$; Lorcaserin x Nicotine interaction, $F(1, 9) = 5.23$, $p = 0.048$). Post-hoc analyses indicated that lorcaserin pretreatments significantly lowered nicotine-enhanced responding on the CR lever compared to the nicotine test. The modest reduction in responding for conditioned reinforcement by lorcaserin alone was not significant ($p > 0.05$).

While lorcaserin itself did not significantly lower responding for a conditioned reinforcer compared to the baseline saline condition, the small reduction in responding warranted a further test for possible impairments in performing an operant response. In this test, lorcaserin injections were compared to saline on performing the same operant response, this time for a water reinforcer. A paired t-test indicated no effect on this measure (Figure 3B; $t(9) = 1.20$, $p > 0.05$).

Fig. 3

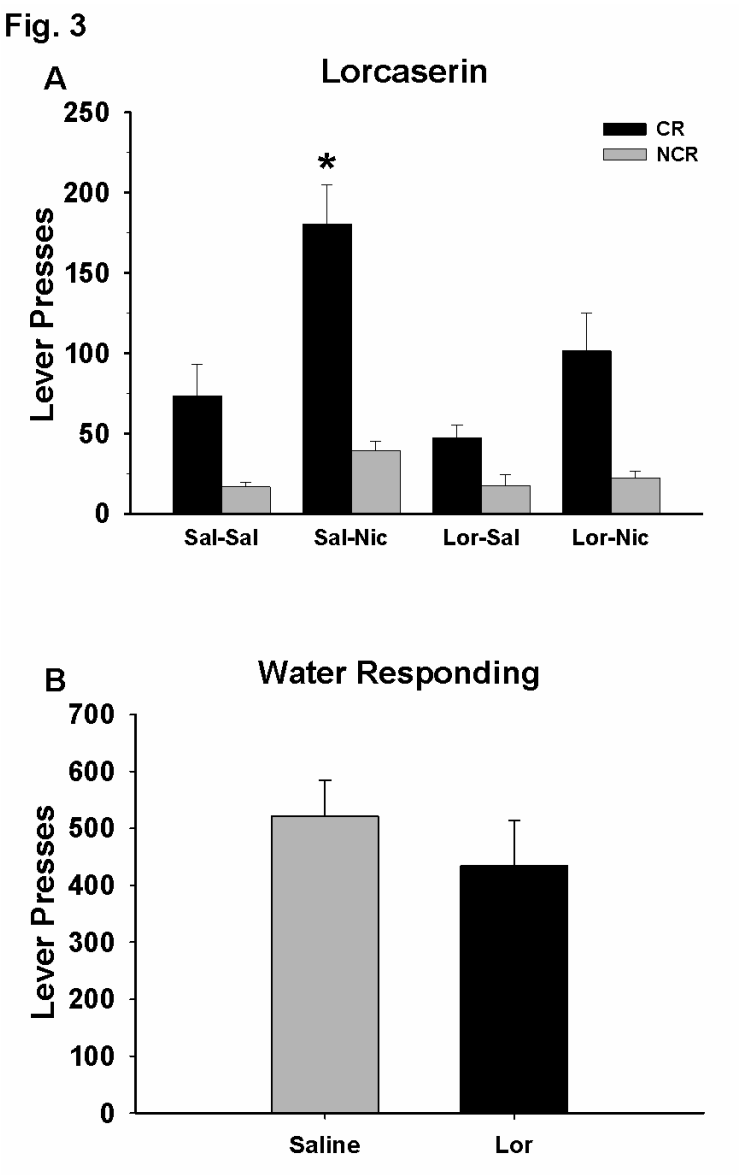

Fig 3. The impact of the 5-HT_{2C} receptor agonist lorcaserin (Lor) on responding for conditioned reinforcement. Panel A shows average (± SEM) response levels on the CR (dark bars) and NCR (grey bars) for each of the four drug treatment conditions where saline (Sal) or nicotine (Nic) injections were preceded by injections of saline (Sal) or lorcaserin, as indicated on the horizontal axis. Panel B shows the average (± SEM) level of responding on a lever paired with the delivery of water. * indicates a significant enhancement in CR-lever responding compared to the Sal-Sal test condition ($p < 0.05$)

3.4 Naltrexone

As depicted in Figure 4, animals showed preferential responding on the CR lever (main effect of Lever, $F(1, 10) = 67.84$, $p < 0.001$) and nicotine enhanced this effect (main effect of Nicotine, $F(1, 10) = 16.96$, $p = 0.002$; Nicotine x Lever interaction, $F(1, 10) = 15.58$, $p = 0.003$). There was no main effect of Naltrexone on CR-directed operant behavior ($F(1, 10) = 2.04$, $p > 0.05$), nor did Naltrexone significantly alter the effect of nicotine on responding for the CR (Lever x Drug x Nicotine interaction, $F(1, 10) = 2.04$, $p > 0.05$). However, further analysis revealed that naltrexone did reduce the response-potentiating effect of nicotine to levels observed for the saline condition ($p > 0.05$), although this reduction of CR lever responding was not significantly lower than levels observed during the nicotine test.

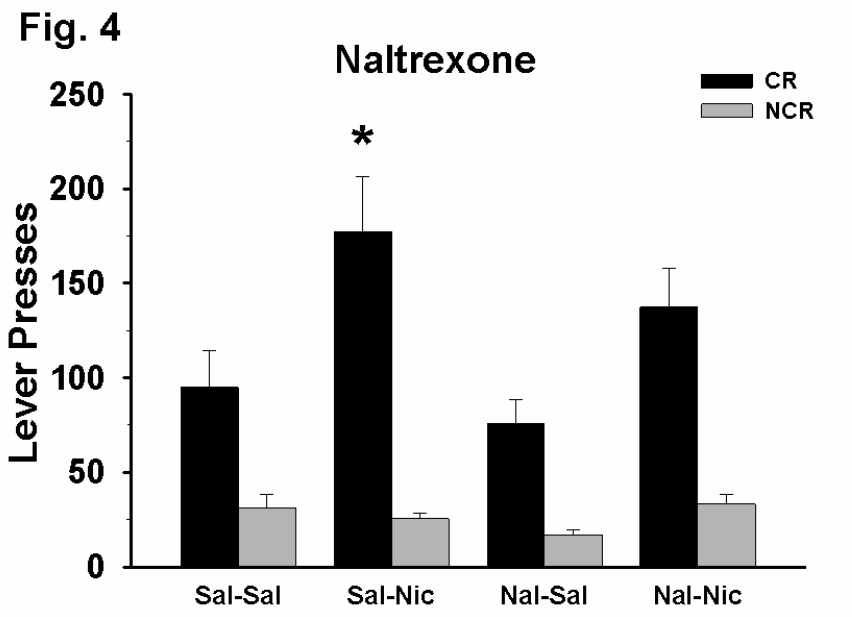

Fig. 4. The effect of administering the *mu*-opioid antagonist naltrexone (Nal) on responding for conditioned reinforcement. Bars depict the mean (± SEM) number of responses on the CR (dark bars) and NCR (grey bars) for each of four tests to assess the impact of saline (Sal) or naltrexone pretreatments on responding for conditioned reinforcement under the influence of Sal or nicotine (Nic).

4 Discussion

These four experiments generated a mixed profile of effects of several drugs used, or could be used, as smoking-cessation aids on nicotine-enhanced responding for a conditioned reinforcer. First, varenicline and lorcaserin were the only agents to significantly reduce nicotine-enhanced responding for conditioned reinforcement. In contrast, the high dose of bupropion increased baseline responding for conditioned reinforcement, and magnified the effect of nicotine on this behavior. Naltrexone itself did not alter responding for conditioned reinforcement, but did modestly decrease the response-potentiating effect of nicotine to similar levels observed in the saline condition. However, that reduction in responding did not significantly differ from levels observed under the influence of nicotine alone. Collectively, these data suggest drugs that aid, or may aid, in smoking cessation can have their effect through disparate neurobehavioral mechanisms, which may interact with the motivating properties of reward-paired CSs.

The variable effects of the four compounds examined in the test of responding for conditioned reinforcement can be, at least partially, explained by their interactions with DA transmission within mesolimbic brain regions. Enhanced dopaminergic neurotransmission has been repeatedly implicated in invigorating responding for conditioned reinforcers (Beninger, Hanson, & Phillips, 1980; Robbins, 1975, 1978; Taylor & Robbins, 1984; Taylor & Robbins, 1986), and dopaminergic projections from the VTA to the NAc are critical to this effect (Parkinson, Olmstead, Burns, Robbins, & Everitt, 1999). Nicotine modulates DA release both directly in the nucleus accumbens (NAc; Threlfell et al., 2012) and through its action on projection neurons in the VTA to the NAc (Di Chiara & Imperato, 1988; McGranahan, Patzlaff, Grady, Heinemann, & Booker, 2011; Ortells & Barrantes, 2011; Vezina et al., 2007). Each of the drugs examined also effect dopaminergic functioning within the VTA-NAc pathway. The effect of these various drugs to upregulate or downregulate mesolimbic DA activity, and their interaction with the effect of nicotine on this activity, seem to be reflected in our measures of responding for conditioned reinforcement.

In this study, varenicline enhanced responding for a conditioned reinforcer by itself, but fully antagonized the ability of nicotine to enhance responding for such reinforcement. Other reports examining the effect of varenicline on nicotine-enhanced responding for reinforcing stimuli show similar behavioral profiles. For example, similar doses of varenicline used in this study

have been shown to decrease brain stimulation reward thresholds when administered alone, but increase brain stimulation reward thresholds when administered prior to nicotine (Spiller et al., 2009). Administration of the 1mg/kg dose of varenicline also shows a trend towards enhancing reinstatement of nicotine self-administration behavior by a nicotine-associated cue, but fully blocks reinstatement by a cue and nicotine prime (O'Conner et al., 2010). Similarly, in a recent report, varenicline enhanced responding for a reinforcing visual stimulus, but blocked the ability of nicotine to enhance such responding (Levin et al., 2012). These behavioral results reflect the data on varenicline-nicotine interactions in mesolimbic DA responses, and its presumed relationship to invigorating responding for conditioned reinforcement (Brunzell et al., 2006; Grottick et al., 2000; Guy & Fletcher, 2012; Rollema et al., 2007a; Rollema et al., 2007b). Studies characterizing the neurophysiological profile of this drug on mesolimbic DA output indicate that varenicline co-administered with nicotine also decreases mesolimbic DA release to levels lower than either drug administered alone, although this difference was not statistically significant (Rollema et al., 2007b). Thus, varenicline, a partial-agonist at the αβ2 nAChRs (Mihalak et al., 2006), may have its therapeutic effect by partially mimicking some of nicotine's motivation-enhancing properties, but fully antagonizing the ability of nicotine to have such an effect (Rollema et al., 2007b). This partial-agonist property may mimic nicotine's pharmacological effect during abstinence to reduce cravings, but antagonize nicotine's effects if nicotine intake is resumed.

Bupropion inhibits DA reuptake (Nomikos, Damsma, Wenkstern, & Fibiger, 1992; Warner & Shoaib, 2005), and so the increase in responding for conditioned reinforcement with the high dose of bupropion in this study is perhaps not surprising, given that other drugs with this action (e.g. amphetamine and pipradrol) have a similar effect (Beninger et al., 1980; Robbins, 1975; Taylor & Robbins, 1984). Our findings are consistent with results showing bupropion enhanced operant responding for a reinforcing visual stimulus, and additively increased nicotine-enhanced responding for that reinforcer (Palmatier et al., 2009). Others have indicated that doses of bupropion within the range used in our study either had no effect, or enhanced the self-administration of nicotine (Rauhut, Neugebauer, Dwoskin, & Bardo, 2003; Shoaib et al., 2003); although some have shown a decrease in nicotine self-administration (Glick, Maisonneuve, & Kitchen, 2002). Doses of bupropion that exceed those used in this study decrease nicotine self-

administration (Rauhut et al., 2005, 2003) and food-maintained responding (Rauhut et al., 2005), suggesting an inverted-U dose-response curve for this drug on motivated behavior.

It has been argued that the dopaminergic agonist-like effect of bupropion aids in smoking cessation by eliminating some of the anhedonia associated with tobacco cessation. Compromised reward processing has been associated with nicotine-withdrawal, and may reflect decreases in mesolimbic DA levels (Cryan, Bruijnzeel, Skjei, & Markou, 2003; Epping-Jordan, Watkins, Koob, & Markou, 1998; George & O'Malley, 2004; Hildebrand, Nomikos, Hertel, Schilstrom, & Svensson, 1998; Warner & Shoaib, 2005; Weaver et al., 2012). As a result, individuals may find smoking abstinence easier to maintain due to a possible reversal of withdrawal-associated DA release. However, it is concerning that bupropion itself *increased* lever pressing for the conditioned reinforcer, and additively interacted with nicotine on this measure of motivation. The results of such motivational enhancements may result in undesired increases in the motivating properties of nicotine-associated stimuli or enhancing the invigorating properties of nicotine itself, both of which could encourage nicotine intake. Such motivational enhancements may indicate why some individuals show a paradoxical increase in tobacco consumption with bupropion (Cousins, Stamat, & de Wit, 2001; Zernig et al., 2004), and partially explain the marginal success rates of this pharmaceutical intervention (George & O'Malley, 2004; Gonzales et al., 2013).

The selective 5-HT_{2C} receptor agonist lorcaserin reduced nicotine-enhanced responding for conditioned reinforcement, and modestly lowered response levels when administered alone. However, such effects were likely not due to response impairments, as the ability to respond for water was not affected by lorcaserin administration. This effect on the motivating properties of the conditioned reinforcer is in accord with a role for 5-HT_{2C} receptor agonists in reducing mesolimbic DA output (Fletcher, 1996; Higgins & Fletcher, 2003; Zaniewska, McCreary, & Filip, 2009) and prior reports implicating this receptor subtype in decreasing responding reinforced by both drug and natural reward-paired CSs (Fletcher, Korth, Robinson, & Baker, 2002; Neisewander & Acosta, 2004). This interaction with nicotine is consistent with findings that lorcaserin reduces nicotine self-administration, and interferes with the reinstatement of responding for nicotine by a nicotine CS (Higgins, et al., 2012; Levin et al., 2011). Presently, lorcaserin is only marketed as an aid for weight loss and to date has not been formally examined as a treatment for smoking cessation. Given that both food and drug reward-directed motivation

can be influenced by similar neuronal circuitry (Berridge, 1996; Berthoud & Morrison, 2008), it is possible that this drug decreases the motivation for both food and drug intake through a common mechanism of blunting the motivating properties of reward-associated cues (Higgins, Silenieks, & Lau, 2012). It is also notable that one of the unwanted side effects of smoking cessation is weight gain (Williamson et al., 1991). Thus, the use of this drug as an appetite suppressant in abstaining smokers may serve the added benefit of attenuating reactivity to smoking-associated cues (Higgins et al., 2013). These two beneficial effects could encourage the individual as he or she works towards attaining a healthier lifestyle.

In this study, *mu*-opioid receptor antagonism with naltrexone did not significantly alter responding for a CR, but it did appear to modestly reduce the response-potentiating property of nicotine on this behavioral measure to a level intermediate between the Sal-Sal and Sal-Nic treatment conditions. The stimulation of *mu*-opioid receptors enhances mesolimbic DA release (Di Chiara & Imperato, 1988; Spanagel, Herz, & Shippenberg, 1990), partially through a presynaptic inhibition of inhibitory GABAergic projections onto dopaminergic cell bodies (Spanagel & Weiss, 1999). Thus, the pharmacological blockade of these receptors may reduce mesolimbic DA activity. However, *mu*-opioid stimulated DA release may not necessarily confer enhanced responding for conditioned reinforcers. The selective stimulation of *mu*-opioid receptors does not alter responding for a conditioned reinforcer, and it is argued that activation of these receptors is more integral to hedonic processing during reward consumption, rather than processing the motivational significance of CSs (Cunningham & Kelley, 1992; Kelley et al., 2002). Accordingly, we also did not observe any significant alterations in responding for the conditioned reinforcer by naltrexone alone, supporting evidence indicating that opiate receptors are not integral to this process (Berridge, 1996; Berridge & Robinson, 1998; Cunningham & Kelley, 1992; Kelley et al., 2002).

In contrast to these reports, others have found a role for *mu*-opioid receptors in cue-induced reinstatement by a drug-paired CS. Liu et al. (2009) found that naltrexone reduced reactivated nicotine-seeking behavior by a drug paired CS following the extinction of operant responding. Furthermore, this same drug has been found to reduce cue-evoked methamphetamine and alcohol-seeking behaviors (Anggadiredja, Sakimura, Hiranita, & Yamamoto, 2004; Ciccocioppo, Martin-Fardon, & Weiss, 2002). In this study, the overall effect of naltrexone on responding for conditioned reinforcement was not significant, although post-hoc comparisons indicated that it

did modestly reduce nicotine-enhanced responding for a conditioned reinforcer to a level that did not differ from saline, suggesting some involvement of *mu*-opioid receptors in this effect.

That naltrexone only altered response enhancements by nicotine may reflect a difference between drug-elicited motivations compared to motivation for a natural reinforcer, such as food or water. In our study and that by Cunningham and Kelley (1992), the CS was paired with a food or drug reward and access to such reinforcement was restricted in the home cages during testing. Perhaps in rats, stimuli associated with reinforcement that fulfills some metabolic need, such as food or water, elicit stronger approach responses due to their salience as a signal for the availability of a necessary resource. This behavior may not be affected by opioid stimulation in the drug-free state. In contrast, enhanced incentive motivation by psychostimulant administration may be partially opiate-dependent, and thus subject to modulation by naltrexone. Evidence for such a profile has encouraged the use of naltrexone in clinical trials because it leaves motivation for non-pharmacological rewards seemingly unaltered. However, our results indicate only a modest, somewhat ambiguous effect of this drug on nicotine-enhanced motivational processes, which may partially explain the mixed efficacy of naltrexone in clinical trials for smoking cessation (David et al., 2009).

4.1 Concluding Remarks

The results of these experiments suggest several conclusions. First, drugs that directly or indirectly modulate dopaminergic neurotransmission can be used to modify the effects of nicotine on responding for a conditioned reinforcer. Whether these drugs enhance or attenuate mesolimbic DA release is reflected by enhanced or blunted responding for such reinforcement. Thus, responding for conditioned reinforcement can be a useful measure of this pharmacological effect of nicotine and interactions with this response. Additionally, these studies suggest responding for conditioned reinforcement may be useful for identifying possible therapeutic indications, or lack thereof, of these drugs on the motivating properties of CSs associated with tobacco consumption in human smokers.

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Chapter 7
General Discussion

1 Overview and Summary of Results

The first aim of this thesis was to characterize the effects of administering nicotine prior to each Pavlovian conditioning trial on Pavlovian approach behavior, and the effect of such nicotine exposure on the ability of nicotine to enhance responding for a conditioned reinforcer (Chapter 3). A second goal was to examine the effect of nicotine administration on approach behavior toward the reward delivery receptacle, or directed at the CS itself when administered during the early versus late phase of Pavlovian conditioning (Chapter 4). Another major focus was to determine the neurochemical underpinnings of this behavior. Toward this goal, the impact of administering selective receptor antagonists at α4β2 or α7 nAChRs (Chapter 3); DA D_1 or D_2 receptor antagonists (Chapter 5); a 5-HT_{2A} receptor antagonist or 5-HT_{2C} receptor agonist on nicotine-enhanced responding for conditioned reinforcement were examined (Chapter 5). The fourth and final aim was to assess the effects of administering four pharmaceuticals: varenicline, lorcaserin, bupropion, or naltrexone, on the ability of nicotine to enhance responding for conditioned reinforcement (Chapter 6). The results of these studies are first briefly discussed, and then interpretations of the findings and their implications are presented.

1.1 Nicotine-induced enhancement of responding for conditioned reinforcement in rats: Role of prior nicotine exposure and α4β2 nicotinic receptors.

As detailed in Chapter 3, nicotine injections given immediately prior to each Pavlovian conditioning session resulted in enhancements in discriminated conditioned approach behavior compared to animals that received saline injections, measured by the proportion of total responses in the reward delivery receptacle during the 5s CS presentation period compared to the 5s period prior to the onset of the CS. All rats learned to respond for the CS as a conditioned reinforcer, with no differences between the saline and nicotine-exposed groups in responding for conditioned reinforcement in a drug-free state. However, animals exposed to nicotine during the Pavlovian phase subsequently displayed increased responding for the CS as a reinforcer when nicotine injections were administered prior to these tests of operant responding. Animals that

received saline injections prior to Pavlovian conditioning trials did not show this enhancing effect of nicotine. Responses on the reinforced lever diminished with repeated testing in the drug-free state, but animals still preferentially responded on the reinforced lever. This pattern of responding did not differ between nicotine or saline exposed groups. The administration of a nicotine injection after animals underwent the repeated testing phase enhanced responding on the reinforced lever in both groups. However, the number of responses on the lever paired with the conditioned reinforcer after this nicotine challenge was higher for the animals that received nicotine during conditioning. Finally, to identify the specific nAChR mediating the effect of nicotine to enhance responding for conditioned reinforcement, the effects of administering the broad-spectrum nAChR receptor antagonist mecamylamine, the α4β2 receptor antagonist DHβE, or the α7 receptor antagonist MLA on nicotine-enhanced responding for conditioned reinforcement were examined in separate groups of animals. These tests revealed that mecamylamine or DhβE, but not MLA, blocked the effect of nicotine, indicating that α4β2 nAChRs, but not α7 receptors, were critical to the response-potentiating effects of nicotine.

1.2 The Effects of Nicotine Exposure During Pavlovian Conditioning in Rats on Several Measures of Incentive Motivation for a Conditioned Stimulus Paired with Water

Experiments in Chapter 4 showed that exposure to nicotine during the Pavlovian conditioning phase resulted in later nicotine-induced enhancements in responding for conditioned reinforcement, but this effect did not depend on whether nicotine was administered early, late, or throughout the Pavlovian conditioning phase. Again, saline-exposed animals did not show this response-potentiating effect with nicotine challenges. Different nicotine exposure schedules also did not affect the extinction of responding on the reinforced lever when the conditioned reinforcer was removed. In all groups, the conditioned reinforcer reinstated operant responding after this extinction period. Co-administration of nicotine and the conditioned reinforcer further enhanced responding, but only in animals with a history of nicotine exposure during conditioning.

In this study, the effect of nicotine to enhance discriminated approach behavior that was previously found in Experiments 1 and 2 of Chapter 3 was seemingly not replicated. However, only one measure of conditioned approach behavior, nosepokes recorded in the water receptacle,

was measured. It was hypothesized that other conditioned approach behaviors may have been enhanced that were not captured by examining approach responses in the water receptacle. Since the design of the initial Pavlovian conditioning study did not allow for measures of other approach responses, such as conditioned approach responses directed towards the CS itself, a follow up experiment was designed. In this additional experiment, an autoshaping procedure was used in order to measure contact with the CS itself. Results showed that nicotine enhanced engagement with a lever-CS, but only if animals were exposed to nicotine during the initial autoshaping trials. The introduction of nicotine in the later trials did not significantly alter engagement with the lever-CS during autoshaping. The lever-CS reinforced a novel operant response in all groups regardless of nicotine history, and an acute challenge with nicotine increased this responding, but only for animals exposed to nicotine during the autoshaping phase. Again, responding for conditioned reinforcement was not altered by the acute administration of nicotine in the saline controls.

1.3 Effects of Dopamine Receptor Antagonists, a 5-HT_{2C} Receptor Agonist, or the 5-HT_{2A} Receptor Antagonist on Nicotine-Induced Enhancement of Responding for Conditioned Reinforcement in Rats

Experiments described in Chapter 5 showed that, administration of the D_1 receptor antagonist SCH 23390, the D_2 antagonist eticlopride, or the 5-HT_{2C} receptor agonist Ro 60-0175 reduced responding for the conditioned reinforcer, and the ability of nicotine to enhance this behavior. However, administration of the 5-HT_{2A} antagonist M100907 did not affect this response-potentiating effect of nicotine. Thus, these experiments implicate DA in mediating the effects of nicotine on responding for conditioned reinforcement, and show that the response can also be altered by manipulating 5-HT function.

1.4 An Examination of the Effects of Varenicline, Lorcaserin, Bupropion, or Naltrexone on Responding for Conditioned Reinforcement in Nicotine-Exposed Rats

The experiments detailed in Chapter 6 tested the effects of four drugs that have some therapeutic indications on nicotine-induced responding for conditioned reinforcement. The four drugs were varenicline, lorcaserin, bupropion, and naltrexone, each of which has distinct pharmacological

actions that may modify the impact of nicotine on responding for conditioned reinforcement. Varenicline, an α4β2 nAChR partial agonist, increased responding for conditioned reinforcement when administered alone, but fully antagonized the effect of nicotine on this behavior. Lorcaserin, a 5-HT_{2C} receptor agonist, reduced responding for conditioned reinforcement, and blocked the effect of nicotine on this response. In contrast, a 30 mg/kg dose of the DA and NE reuptake inhibitor bupropion increased responding for conditioned reinforcement, and further increased the response-potentiating effect of nicotine. Finally, naltrexone modestly reduced nicotine-enhanced responding for conditioned reinforcement, but did not significantly lower response levels compared to those exhibited under the influence of nicotine alone.

2 Interpretation and Possible Implications of Findings

2.1 Possible Role for Sensitization in Mediating Nicotine-Enhanced Responding for Conditioned Reinforcement

As detailed in Chapters 3 and 4, exposure to nicotine during Pavlovian conditioning sessions when the CS-US pairings were experienced, did not affect subsequent operant responding for the CS as a conditioned reinforcer in a drug-free state. In addition, the timing of nicotine exposure during Pavlovian conditioning (i.e., either prior to the initial trials, during the learning phase, or prior to the later trials, during the maintenance phase) did not affect the ability of nicotine to later enhance responding for conditioned reinforcement. It was also shown that altered Pavlovian approach behavior was not necessary for nicotine to increase responding in the operant phase of these experiments. Together, these data suggest that any nicotine-induced alterations in learning about the motivational significance of the CS during Pavlovian conditioning do not affect the ability of that CS to acquire conditioned reinforcing properties and support the acquisition of a novel operant response.

The effect of nicotine injections administered during the Pavlovian conditioning phase to confer enhancements in responding for conditioned reinforcement following an acute nicotine injection may be due to a sensitization effect. In other settings, five days of exposure to an injection of nicotine prior to locomotor testing is sufficient to induce a sensitized locomotor activity response to a challenge injection with nicotine (Govind, Vezina, & Green, 2009; Reid, Ho, & Berger, 1996). In another report where the effects of nicotine administration on responding for a light visual stimulus reinforcer were examined, approximately three days of operant testing, preceded

by nicotine or saline injections, were necessary for the nicotine-exposed animals to show enhanced responding compared the animals that received the same number of saline injections (Palmatier et al., 2007). Subsequent trials testing the effect of nicotine on responding for the visual stimulus indicated that further exposure to nicotine continued to increase differences in response levels between the two groups. Furthermore, this effect of nicotine to enhance responding reinforced by a visual stimulus was not due to context-dependent associations between nicotine and the stimulus, but rather depended on exposure to the drug itself. In a separate experiment, two groups of animals were exposed to nicotine injections, one group received nicotine injections before operant testing and the other group was administered the same number of nicotine injections, but after operant conditioning sessions. Both groups displayed the same number of responses for the visual stimulus when challenged with an acute nicotine injection (Palmatier et al., 2007). In Chapter 4, we found that 7 days of nicotine administration prior to Pavlovian conditioning, regardless of when injections were administered during the conditioning phase, was sufficient to enhance responding for a conditioned reinforcer under the acute effects of nicotine. This also suggests a sensitization-like effect, as nicotine-induced alterations in conditioning were not necessary for a later nicotine challenge injection to enhance responding for conditioned reinforcement.

The results from this thesis indicate that nicotine-induced alterations in Pavlovian approach behavior are not necessary for the development of later nicotine-enhanced responding for conditioned reinforcement. Since it was not found that the schedule of nicotine exposure was important for nicotine-enhanced responding for conditioned reinforcement, and findings from other studies support a sensitization-like effect on behavior following repeated nicotine injections, the combined results imply that incentive stimuli do not have to be previously associated with nicotine administration for nicotine to induce a reinforcement-enhancing effect. Thus, prior experience with nicotine may result in later nicotine-induced enhancements in incentive motivation for a wide range of reward stimuli that may have not been previously paired with nicotine administration. In the context of human smoking behavior, perhaps repeated nicotine use enhances both the motivating properties of stimuli previously associated with nicotine consumption (Rose & Levin, 1991), as well as the motivating properties of reinforcing stimuli that have never been paired with nicotine consumption (Martin-Solch, Magyar, Kunig, Missimer, Schultz, & Leenders, 2001; Dawkins, Powell, West, Powell, & Pickering, 2006).

These effects may contribute to the perpetuation of smoking behavior, or further escalations in tobacco use, as smoking is reinforced in novel contexts with new reinforcers.

2.2 Nicotine-enhanced Responding for Conditioned Reinforcement: Possible Role of Sensitized Mesolimbic DA Function

The effect of nicotine to enhance responding for conditioned reinforcement may result from sensitization of nicotine-facilitated DA release in mesolimbic regions, which occurs with repeated exposure to nicotine (Balfour et al., 1998; Di Chiara, 2000; Govind et al., 2009). Prior reports have indicated that just five daily subcutaneous injections of nicotine (0.4 mg/kg) are sufficient to sensitize mesolimbic DA release, as measured using *in vivo* microdialysis, in response to acute nicotine administration (Balfour et al., 1998). This exposure schedule corresponds with the level of exposure necessary to sensitize locomotor responses to an acute nicotine injection, and the number of nicotine injections necessary to enhance the reinforcing properties of non-drug stimuli (Balfour et al., 1998; Palmatier et al., 2007; Chapter 4). Furthermore, in a previous study of the effects of repeated nicotine exposure administered prior to any behavioral tests on responding for conditioned reinforcement, nicotine-exposed animals displayed significantly larger increases in responding for conditioned reinforcement compared to saline-exposed animals following an intra-accumbens infusion of amphetamine (Olausson et al., 2004b). This sensitized response to amphetamine, a dopamine reuptake inhibitor and releaser, suggests that nicotine exposure induces changes in mesolimbic dopamine functioning that render these DA cells hyperexcitable to agents that stimulate DA release (Benwell & Balfour, 1992).

Sensitization of mesolimbic DA functioning resulting from repeated nicotine injections may be due to changes in nAChR sensitivity, specifically at receptors containing α4 or β2 subunits. Several studies have shown that nicotine binding at nAChRs containing the α4, β2, or both subunits enhances mesolimbic dopamine release, and mediates the behavioral activating properties of nicotine with repeated exposure (Brunzell et al., 2006; Tapper et al., 2004; Threlfell et al., 2012), including responding for conditioned reinforcement (Chapter 3). The α4β2 nAChRs are localized throughout the cortex, including the mesolimbic regions involved in reward processing and incentive motivation (Kelley, 2004; Picciotto, Caldarone, King, & Zachariou, 2000; Robinson & Berridge, 1993). These receptors are located on both

dopaminergic cell bodies and GABAergic interneurons within the VTA (Markou, 2008; Rollema et al., 2007), as well as on DA axons within the NAc (Picciotto et al., 2000; Threlfell et al., 2012).

Nicotine enhances NAc dopamine release when it binds to the α4β2 nAChRs by several possible mechanisms. One possible mechanism is by the stimulation of α4β2 nAChRs located on DA cell bodies within the VTA (Markou et al., 2008). Intra-VTA stimulation of nAChRs enhances mesolimbic DA cell firing (Ferrari et al., 2001), and mice lacking the *β2 subunit do not display enhanced intra-accumbens DA release in response to an injection of nicotine (Picciotto et al., 1998), suggesting a mediating role for these receptors in nicotine-induced mesolimbic DA release. A second possible mechanism of enhanced DA release upon nicotine administration is a reduction in α4β2 receptor-mediated stimulation of GABAergic neurons in the VTA (Rollema et al., 2007). These high-affinity receptors rapidly desensitize under the acute effects of nicotine, thereby attenuating GABA-mediated inhibition of DA release (Govind et al., 2009). Third, a recent study has shown that α4β2 receptors localized to the dopaminergic axons in the NAc may also control mesolimbic DA release, and may do so independently from neuronal activation within the VTA (Threlfell et al., 2012).

Repeated exposure to nicotine may affect all three mechanisms of nicotine-mediated mesolimbic DA release. Repeated nicotine administration is associated with an upregulation of α4β2 nAChRs in mesolimbic brain regions (Govind et al., 2009; Ortells & Barrantes, 2011). *In vitro* evidence suggests this α4β2 nAChR upregulation may occur across multiple cell types, including dopaminergic and GABAergic neurons within the VTA and NAc (Govind et al., 2009). An upregulation of α4β2 nAChRs located on the dopaminergic cell bodies of neurons projecting from the VTA to the NAc may render these receptors hyperexcitable to subsequent stimulation. Alternatively, it has been reported that the upregulation of VTA α4β2 nAChRs following repeated nicotine exposure is primarily localized to the GABAergic cells (Nashmi et al., 2007). It is hypothesized that this increase in receptor availability is due to a post-translational mechanism that enhances nicotine binding affinity at these receptors (Govind et al., 2009). The result of an enhanced affinity for nicotine at the α4β2 nAChRs localized to the GABAergic neurons could be an expedited desensitization response. Rapid desensitization of α4β2 nAChRs on GABAergic neurons would reduce GABA-mediated inhibition of DA neurons, thereby disinhibiting DA cell firing within the VTA and enhancing mesolimbic DA release (Govind et

al., 2009). A third possible mechanism of enhanced mesolimbic DA release is a sensitized response to α4β2 receptor stimulation within the NAc. An upregulation of those α4β2 nAChRs located on the dopaminergic axons in the NAc that enhance the intra-accumbens DA response could also contribute to the sensitized mesolimbic DA response following repeated nicotine exposure (Threlfell et al., 2012).

2.2.1 Possible Mechanisms by Which a Sensitized Mesolimbic DA response to Repeated Nicotine Administration Invigorates Responding for Conditioned Reinforcement

As previously described (Introduction, Chapters 5, 6), the administration of psychostimulant drugs, such as the dopamine reuptake inhibitor and releaser amphetamine, or the administration of agonists at the DA receptors themselves increase responding for conditioned reinforcers (Beninger, Hanson, & Phillips, 1980; Robbins, 1975; Taylor & Robbins, 1984). Nicotine-induced increases in mesolimbic DA release also likely underlies the ability of nicotine to invigorate such responding. However, the sensitization of drug-induced DA release following repeated injections is also associated with enhancements in locomotor activity (Balfour, Wright, Benwell, & Birrell, 2000; Govind et al., 2009; Kalivas & Stewart, 1991). While locomotor behavior was not measured here, it is unlikely that such a general increase in behavioral output accounted for the effect of nicotine on responding for conditioned reinforcement. Response enhancements were observed only on the reinforced (CR) lever following nicotine administration. Responding on the non-reinforced (NCR) lever did not change under the influence of a nicotine injection compared to saline. Furthermore, responding for the light/tone stimulus was not altered by nicotine administration when that stimulus was explicitly unpaired with reinforcement during the conditioning phase (Chapter 4). The absence of an effect of nicotine administration to alter responding for this unconditioned stimulus is consistent with other reports indicating that the administration of nicotine or other psychostimulants selectively enhance responding for CSs, and do not affect responding for unconditioned stimuli (Chaudhri et al., 2006; Taylor & Robbins, 1984). Such specific effects on responding for reward-paired stimuli would not be consistent with a general response enhancement, suggesting that enhanced responding for conditioned reinforcement captured selective enhancements of incentive motivation following nicotine administration.

A sensitised mesolmbic DA system that develops following repeated nicotine exposure may affect the neural response to salient, reward-predictive stimuli (i.e., CSs). The presentation of a salient stimulus predictive of reinforcer delivery results in a different dopaminergic response from the presentation of a stimulus that has never been paired with reinforcement (Horvitz, Choi, Morvan, Eyny, & Balsam, 2007). The presentation of reward-conditioned stimuli results in enhanced dopaminergic cells firing within the VTA during the presentation of the stimulus, which continues for several hundreds of milliseconds following its presentation. In contrast, the presentation of salient, non-reward stimuli results in a short, rapid burst of VTA DA cell firing that is quickly followed by an inhibition of dopaminergic activty. The dopamergic response to reward stimuli is hypthesized to promote neuroplastic changes that connect the presentation of the stimulus with the availability of reinforcement (Horvitz et al., 2007; Sutton & Beninger, 1999), and magnitude of mesolimbic dopaminergic activity functions as a "teaching signal" (Hollerman & Schultz, 1998). Over time, as the incentive salience of the stimulus is learned, presentations of the CS, rather than the reinforcer, are associated with mesolimbic DA release, and this response is associated with the expression of reward-seeking behaviors (Roitman, Stuber, Phillips, Wightman, & Carelli, 2004). Since stimuli that have not been paired with reward are not associated with transient increases in VTA DA cell firing, and do not display this hallmark mesolimbic DA signal (Horvitz et al., 2007), these unpaired stimuli may be unsusceptible to any dopaminergic modulation by nAChR activation. An implication of the disparate VTA DA cell activity in response to reward versus non-reward salient stimuli is that stimuli paired with rewards may be more likely to be modulated by the pharmacological effects of nicotine on DA activity. Further following from this assertion, neutral or unpaired stimuli would be less likely to become conditioned reinforcers of tobacco use.

2.3 Role of Conditioning History on Attraction Toward the CS During Responding for Conditioned Reinforcement

While the timing of nicotine administration during Pavlovian conditioning did not affect levels of nicotine-enhanced responding for conditioned reinforcement among the nicotine-exposed animals, it is possible that conditioning history altered some of the attractive properties of the CS, defined as engagement with the CS when it serves as a conditioned reinforcer (Chapter 4). Animals that received nicotine prior to the initial Pavlovian autoshaping sessions (i.e., the Nicotine Early or Nicotine Throughout groups, Chapter 4), engaged more with the lever when it

was presented as a reinforcer than animals that only received nicotine prior to the final 6 trials. Perhaps nicotine exposure early in conditioning changes the learned motivational significance of the CS as a modifier of conditioned approach behavior, and subsequent exposure to nicotine in these animals reveals an incentive memory that confers invigorated approach behavior towards the CS when it is presented as a reinforcer.

2.3.1 Possible Neural Mechanisms Underlying the Effect of Nicotine to Enhance Engagement with the CS When Serving as a Conditioned Reinforcer

The ability of nicotine exposure during the initial conditioning trials to enhance approach towards the CS when presented as a conditioned reinforcer may be due to alterations in DA signaling induced by nicotine. Evidence suggests that, as the motivational significance of a CS is learned, presentations of that CS during Pavlovian conditioning are associated with mesolimbic DA release (Clark, Collins, Sanford, & Phillips, 2013). This may reflect the role of DA as a "teaching" signal in the early phases of incentive learning (Hollerman & Schultz, 1998). However, following repeated conditioning trials, when the contingency between the CS and the US has been well-established, DA release within the NAc is no longer elicited by presentations of the CS (Clark et al., 2013). In Chapter 4, Experiment 2, nicotine administration prior to the later conditioning trials did not affect later engagement with the CS when presented as a conditioned reinforcer. In contrast, animals that received nicotine in the early conditioning trials showed nicotine-induced enhancements in engagement with the autoshaping CS when it was presented as a reinforcer. These discrepant results may be because CS presentations were likely no longer associated with increased mesolimbic DA release in the later trials (Clark et al., 2013). If mesolimbic DA release represents a teaching signal, this suggests that the learned motivational significance of the CS had already been established, and was not subject to further manipulation by pharmacological effects of nicotine administration.

2.3.2 Implications of an Effect of Nicotine to Alter the Motivational Significance of Reward Stimuli, and Enhance Attraction Toward CSs

It has been suggested that the incentive salience of CSs may affect approach behavior in at least three ways: they can bias attention (Flagel, Watson, Robinson, & Akil, 2007), they can invigorate ongoing approach behaviors (Berridge, Robinson, & Aldridge, 2009), or they can

support new learning by functioning as conditioned reinforcers (Berridge et al., 2009; Mackintosh, 1974; Saunders & Robinson, 2013). In relation to addiction, these components of incentive motivation may all affect continued drug use and relapse, but only the latter is measured in the test of responding for conditioned reinforcement. Considering the preliminary evidence that nicotine exposure during the learning phase of a Pavlovian autoshaping procedure results in nicotine-enhanced approach and engagement with the lever-CS as a conditioned reinforcer, it is possible that nicotine alters the learned motivational significance of CSs associated with its pharmacological effects, and biases attention toward them. These data also suggest that CSs that acquire their motivational significance under the influence of nicotine are more likely to bias attention compared to stimuli that gained motivational significance without an influence of nicotine. Hence, CSs that are particularly related with nicotine consumption, and were not encountered prior to nicotine use, may be particularly strong captivators of attention. A clinical implication is that those stimuli may be the most prominent conditioned reinforcers of tobacco smoking behavior.

2.4 The Utility of Responding for Conditioned Reinforcement as a Behavioral Indicator of Pharmaceutical Interference with Nicotine's Effects on the Reinforcing Properties of Reward-Associated Cues

The results in this thesis showed that dopamine receptor activation is critical to the effect of nicotine to enhance responding for conditioned reinforcement (Chapter 6). In addition, these studies indicated that the stimulation of 5-HT_{2C} receptors can interact with the ability of nicotine to enhance this behavior (Chapter 6). Another important finding is that the test of responding for conditioned reinforcement can be modulated by drugs approved for tobacco cessation, and other pharmaceuticals with possible therapeutic indications for curbing tobacco use (Chapter 7). As previously described (Discussion, Chapter 7), the effect of varenicline and lorcaserin to reduce nicotine-enhanced responding for conditioned reinforcement, and the ability of bupropion to increase this behavior, reflects the properties of these drugs to reduce, or facilitate, mesolimbic DA output, which is likewise enhanced by nicotine. An implication of this finding is that responding for conditioned reinforcement may serve as a behavioral measure of drug-induced mesolimbic DA activity related to the presentation of reward-associated CSs. Considering enhancements in mesolimbic DA activity are hypothesized to reflect cue-evoked incentive

motivation (Berridge & Robinson, 1998), this test may have some predictive validity for assessing the effects of various drugs on cue-elicited nicotine cravings, or the effect of these drugs on the ability of nicotine CSs to reinforce smoking behavior. Some studies in human cigarette smokers support this claim. For example, the administration of varenicline, an α4β2 partial agonist that can act as a full antagonist of the reinforcing effects of nicotine, blocks the nicotine-enhanced responding for conditioned reinforcement. It would therefore be predicted that administering varenicline to human smokers would reduce cue-evoked cravings for cigarettes. In one study of human smokers, varenicline was found to reduce craving for cigarettes in the presence of smoking cues. In addition, this same study found that varenicline administration reduces mesolimbic brain activity in the presence of these smoking-associated CSs (Franklin et al., 2011). This effect of varenicline to reduce cue-elicited craving in abstained smokers was replicated in another study (Brandon et al., 2011). Furthermore, this same study assessed the ability of varenicline to reduce reported smoking reward upon the re-initiation of tobacco intake, a measure that may be influenced by the conditioned reinforcing properties of nicotine (Caggiula et al., 2001; Rose et al., 2000). In contrast to varenicline, the administration of bupropion enhances responding for conditioned reinforcement, and enhances the effect of nicotine on this response. While some human studies have yielded beneficial results for bupropion in reducing cue-elicited cravings for cigarettes (Brody et al., 2004), others have indicated that buproprion administration was ineffective in reducing cue-elicited cravings for cigarettes (Ferguson & Shiffman, 2009), and may paradoxically increase smoking behavior in some individuals (Zernig et al., 2004), perhaps by enhancing the influence of conditioned reinforcers of tobacco consumption. One study of naltrexone administration among human smokers has yielded positive results for this drug in reducing cue-elicited cravings to smoke compared to placebo (Hutchison et al., 1999), but quit rates with adjunctive naltrexone have been mixed (King et al., 2006; Wong et al., 1999). Altogether, these clinical effects are seemingly in accord with the effects of these drugs to reduce, enhance, or modestly decrease responding for conditioned reinforcement. Thus, this behavioral procedure may be useful as a preclinical screen to assess the therapeutic potential of drug interventions to reduce cue-elicited cravings, or the conditioned reinforcing effects of nicotine intake.

A role that the conditioned reinforcement procedure could play in future translational research is to provide preclinical evidence that some drugs may be particularly effective in reducing the

reinforcing properties of nicotine-associated CSs. This could potentially improve the implementation of pharmaceutical intervention strategies among smokers that are particularly vulnerable to this one contributing factor of tobacco use. Towards this objective, future work in humans could develop a clinical measure of individual variations in responses to the reinforcement-enhancing properties of nicotine use. A few studies have laid some of the groundwork necessary for such an endeavor to be pursued by indicating that physiological responses to the conditioned reinforcing properties of tobacco consumption show variability, and can be related to subjective feelings of pleasure and reinforcement while smoking (Brody et al., 2009; Kang et al., 2012; Rose et al., 2000). Specific to neurophysiology, one way this has been accomplished is by measuring functional resonance imaging (fMRI) blood-oxygen level dependent (BOLD) responses in mesolimbic brain regions following the consumption of regular compared to denicotinized cigarettes (Brody et al., 2009). However, the Brody et al. (2009) study did not measure an important control response: the effect of nicotine administration in the absence of any CSs on mesolimbic brain activation. This measure would be necessary to develop a more definitive indication of the possible additive effects of nicotine itself on the reinforcing properties of smoking-associated CSs. Future expansions of these studies could compare mesolimbic brain activity, as well as self-reported sensations of reinforcement, in response to (1) the administration of nicotine itself in the absence of any associated cues (e.g., via a transdermal patch or intravenous infusion), (2) the consumption of a denicotinized cigarette (i.e., the "cue-only" condition), or (3) the consumption of a fully-nicotinized cigarette. Subtracting the neural and affective responses to systemic nicotine and the consumption of a denicotinized cigarette from consuming a fully-nicotinized cigarette could provide a neural marker to indicate differential responses to the reinforcement-enhancing properties of nicotine. This information could be used to develop clinical tools to that measure brain responses relating to differences in processing the reinforcement-enhancing effects of nicotine among individual smokers. Variance in this clinical measure could be used to predict the probability of abstinence for various drugs, as informed by the ability of those drugs to reduce nicotine-enhanced responding for conditioned reinforcement. Perhaps smokers that score relatively highly on measures reflecting the reinforcement-enhancing effects of nicotine would be better served by pharmaceutical interventions that are effective in reducing nicotine-enhanced responding for conditioned reinforcement in preclinical studies.

3 Future Directions

Additional questions follow from the results of these experiments that could be addressed in future studies examining the effects of nicotine on responding for conditioned reinforcement. First, the possible effect of nicotine administration prior to the early autoshaping sessions on enhanced engagement with the autoshaping CS when it served as a conditioned reinforcer may be further examined. Second, these experiments produced a large amount of behavioral data regarding the effects of systemic injections of nicotine and various pharmaceuticals. However, the precise brain regions where these drugs interact to produce the observed behavioral effects remain to be determined. A third possible avenue for future research includes examining the effect of nicotine withdrawal in dependent animals on responding for conditioned reinforcement, and possible pharmacological modulation of responding during withdrawal states.

3.1 The Possible Influence of Nicotine Exposure During Pavlovian Association Learning on Nicotine-enhanced Attraction Toward a CS when it Serves as a Conditioned Reinforcer

Animals that received nicotine in the initial autoshaping trials (Nicotine Early and Nicotine Throughout groups, Chapter 4) later showed more engagement with the CS when it was presented as a reinforcer under the acute effects of nicotine, compared to the saline exposed animals, or the animals that received nicotine in the later trials (Chapter 4). I hypothesized that this effect may be due to a nicotine-induced alteration in learning about the relationship between the CS and US in the early trials, or learning phase (Chapter 4). To test the hypothesis that nicotine alters the attractive properties of the CS during the learning phase, future work would involve administering nicotine prior to the first 6 autoshaping sessions, and comparing approach behavior during tests of conditioned reinforcement to a control group that received nicotine in the home cage after the first 6 trials, separated by a substantial time period to avoid any effects on memory consolidation. If nicotine-induced enhancements in attraction toward the CS during the test for conditioned reinforcement occur in those animals that receive injections prior to conditioning, but not in the control group, this result would support a learning effect of nicotine during early conditioning on approach toward reward-predictive stimuli.

3.2 An Examination of Candidate Brain Regions Involved in Nicotine-Enhanced Responding for Conditioned Reinforcement

In this thesis, the likely role of the mesolimbic DA VTA-NAc pathway in nicotine-enhanced responding for conditioned reinforcement was emphasized. Intact DA terminals within the NAc are necessary for nicotine reinforcement in self-administering animals (Corrigall et al., 1992). In tests of responding for conditioned reinforcement, excitotoxic lesions of the NAc, particularly the shell region, block the response-potentiating effects of other psychostimulants, such as amphetamine (Burns et al., 1993; Parkinson et al., 1999). However, the possible mediating role of the NAc in nicotine-enhanced responding for conditioned reinforcement has yet to be assessed. Other candidate brain regions that may also be involved in the response-potentiating effects of nicotine are the AMY and PFC (Burns et al., 1993; Kelley, 2004; Parkinson et al., 1999; Cardinal et al., 2002). However, at least one study suggests that the PFC is not critical to responding for conditioned reinforcement, or its enhancement by the psychomotor stimulant amphetamine (Burns et al., 1993). Likewise, this structure may not be critical for the effects of nicotine on this response. This same study (Burns et al., 1993) also showed that lesions of the AMY did not affect general increases in responding by amphetamine, but did block preferential responding on the reinforced lever. A similar effect of AMY lesions may be observed for nicotine-enhanced responding for conditioned reinforcement.

While both nicotine and amphetamine are stimulants that enhance mesolimbic DA release, they do so by different pharmacological mechanisms. Amphetamine acts as a DA reuptake inhibitor and releaser at the DA terminals (Sulzer, Chen, Lau, Kristensen, Ravport, & Ewing, 1995), while nicotine enhances DA output via nAChR stimulation on the DA cell bodies, or presynaptic modulation of DA release through the stimulation of nAChR on GABAergic and glutamatergic terminals, or on the DA axons themselves (Di Chiara & Imperato, 1988b; Markou, 2008; Picciotto et al., 1998). Considering these different mechanisms of action for nicotine and amphetamine to enhance dopaminergic activity, it cannot be ruled out that selective ablations of the PFC, AMY and NAc differentially modulate nicotine-enhanced responding for conditioned reinforcement when compared to the effects of amphetamine. In order to assess the roles of the PFC, AMY, and NAc in the response-potentiating effects of nicotine, animals would undergo Pavlovian approach training, each session preceded by an injection of nicotine as previously

described in this thesis. Then, prior to tests of responding for conditioned reinforcement, these animals would be divided into three separate groups where the effects of excitotoxic lesions localized to the PFC, AMY, or NAc on nicotine-enhanced responding for conditioned reinforcement would be assessed.

3.3 The Possible Effects of Nicotine Withdrawal on Responding for Conditioned Reinforcement

Several studies have indicated possible deficits in reward functioning following nicotine withdrawal (Cryan, Bruijnzeel, Skjei, & Markou, 2003; Epping-Jordan, Watkins, Koob, & Markou, 1998; Markou & Paterson, 2001), and hypothesized that motivation to avoid of this effect may contribute to relapse in some smokers (Hughes, 2007; Snuggs & Hajek, 2013). Nicotine enhances sensitivity to brain stimulation reward (BSR), and the removal of chronic nicotine administration results in reward-deficits, measured by increases in reward threshold responses. It is thought that these effects are due to decreases in nicotine-modulated dopamine release, mediated by changes in α4β2 receptor availability and sensitivity with chronic nicotine exposure (Fowler et al., 2009; Markou, 2008; Govind et al., 2009). In support, reward deficits caused by acute nicotine withdrawal can be mimicked by administering the α4β2 antagonist DhβE to animals chronically exposed to nicotine via osmotic minipumps delivering 3.16 mg/kg/day of nicotine (Epping-Jordan et al., 1998). The administration of pharmaceutical agents that enhance dopamine transmission, particularly bupropion, reverses this effect (Cryan et al., 2003). Responding for conditioned reinforcement is another purported assay of reward functioning, as such behavior can be enhanced, or reduced, by drugs that increase (Beninger et al., 1980; Robbins, 1975, 1978; Taylor & Robbins, 1984) or decrease (Chapter 5) mesolimbic DA cell activity, respectively. Therefore, responding for conditioned reinforcement may be similarly affected by nicotine withdrawal. To test this hypothesis, animals would be subjected to chronic nicotine infusions via osmotic minipumps. Then, prior to tests of responding for conditioned reinforcement, the animals would undergo precipitated nicotine withdrawal induced by the administration of a nicotinic antagonist, such as mecamylamine. Responding for conditioned reinforcement would then be monitored. If such responding was reduced, this would suggest that the test of responding for conditioned reinforcement may also provide an assay of reward functioning.

4 Final Statements

In total, the experiments in this thesis provide further evidence supporting the substantial role that CSs play in mediating nicotine's reinforcing effects. We extended prior research examining the effects of nicotine on responding for conditioned reinforcement by assessing the role of prior nicotine exposure during conditioning, and identifying a mediating role for the α4β2 nAChR subtype in this behavioral effect. We also found D_1 and D_2 receptor involvement in this effect, and a role for 5-HT_{2C} receptor stimulation in inhibiting nicotine-enhanced responding for conditioned reinforcement. Finally, we examined the impact of several FDA-approved pharmaceuticals on nicotine's effect to enhance responding for conditioned reinforcement. Varenicline and lorcaserin reduced the impact of nicotine on such responding, while bupropion enhanced this behavioral effect. Such results, combined with studies in humans, suggest that this measure of incentive motivation could be used in preclinical studies of the efficacy of pharmaceutical manipulations to alter cue-evoked incentive motivation, which may contribute to the intransigence of human smoking behavior among individuals struggling to quit.

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