NICOTINE ENHANCEMENT OF CONDITIONED RESPONDING: INVOLVEMENT OF THE ORBITOFRONTAL CORTEX

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ABSTRACT

Sierra Jeanel Stringfield: Nicotine enhancement of conditioned responding: Involvement of the orbitofrontal cortex
(Under the direction of Donita Robinson)

Nicotine abuse is a substantial public health problem, and one cause of the resilience of nicotine addiction is the influence of conditioned cues. Repeated pairings of an environmental stimulus with a reward, such as the effects of a drug, can lead to the formation of an association between the now conditioned stimulus and the expected outcome. These stimuli are capable of acquiring incentive properties, in which they become appetitive and wanted and are able to motivate behavior. In humans, exposure to these stimuli can result in the expression of a conditioned response, such as experiencing the subjective feeling of craving after exposure to drug-associated cues and attentional bias toward those cues. Pavlovian conditioned approach can be used to model these stimulus-outcome associations in animals. Animals will show conditioned approach responses, and drugs such as nicotine can increase the expression of this behavior. This dissertation will investigate the influence of nicotine on Pavlovian conditioned approach and the neuronal circuitry that contributes to the expression of these behaviors. Specifically, we investigated the orbitofrontal cortex, a region of the prefrontal cortex that is responsible for representing stimulus-outcome associations and influencing behavioral flexibility. Pavlovian conditioned approach was assessed in rats under normal conditions and tested in situations that were designed to challenge the function of the orbitofrontal cortex, to measure the flexibility of conditioned approach, and how nicotine reduced this flexibility. We also investigated the contributions of sex differences to the expression of this behavior, and the expression of BDNF protein after nicotine exposure or conditioned approach training. We found that nicotine enhances Pavlovian conditioned approach in both males and females, and the

OFC is involved in expression of this behavior. In addition, nicotine reduces the flexibility of conditioned responses after a change in expected outcome, but did not influence BDNF protein expression. These studies as a whole contribute to our understanding of the ability of nicotine to influence the salience of conditioned cues, hopefully advancing treatment options that focus on reducing the motivational hold that conditioned cues have on smokers who are attempting to remain abstinent.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

5CSRTT	5-choice serial reaction time task
BLA	Basolateral amygdala
CeA	Central amygdala
CS	Conditioned stimulus
DA	Dopamine
DLS	Dorsolateral striatum
DMS	Dorsomedial striatum
NAc	Nucleus accumbens
nAChR	Nicotinic acetylcholine receptor
lOFC	Lateral orbitofrontal cortex
mOFC	Medial orbitofrontal cortex
OFC	Orbitofrontal cortex
PFC	Prefrontal cortex
US	Unconditioned stimulus
VTA	Ventral tegmental area

Chapter 1: Introduction

Public health impact of nicotine use

Drugs of abuse present a major health and economic burden on both a global and national scale. In the United States, 27.1 million people (10.1% of the population) age 12 and older report using an illicit drug in the past 30 days, and 8% of the population meets criteria for a substance use disorder. In the year 2014, 21 million people were identified as needing treatment for a substance use disorder, yet only 2.3 million received treatment during the year (Center for Behavioral Health Statistics and Quality, 2016). Nicotine or tobacco use is of particular global health relevance, as it is one of the leading causes of preventable death and severe health consequences. Nearly half a million adults in the United States die prematurely from health problems associated with smoking, and this contributes to the annual economic cost of tobacco use, estimated at $289 billion dollars (United States Department of Health and Human Services, 2014).

Globally, 967 million people smoke (Ng et al., 2014) and in the United States, 40 million people are current smokers (17% of the population, Jamal et al., 2015). While cigarette smoking has declined steadily since 1980, use of electronic vaporizers such as e-cigarettes as a nicotine delivery system has emerged and may account for some of the decrease in traditional cigarette use. For example, in 2013 more than a quarter million students below the age of 18 had used e-cigarettes, but had never smoked a traditional cigarette (Bunnell et al., 2015). A majority of smokers are interested in quitting, with 68% of current smokers expressing a desire to quit. Of those that attempt to quit smoking, only 7.5% reported a recent ability to terminate the behavior (Babb et al., 2017). Unfortunately, greater than 95% of those who attempt to quit without treatment relapse within less than a year (Hughes et al., 2004) and with treatment, 70% may still

relapse within a year (Piasecki, 2006). Numerous treatment options exist for smoking cessation, providing success when used alone or in combination, and these treatments are more successful than attempting to quit without assistance (Patnode et al., 2015). Yet, the rate of continued cessation from smoking is lower than would be desired. While there is a decline in traditional tobacco consumption mechanisms, the advent of newer vaporizers and e-cigarettes present a potential shift in mechanism for consuming nicotine, particularly in youths. Nicotine use has not been eradicated from developed countries, and tobacco use is still a significant health problem in developing countries. Thus, understanding the contributions of nicotine to continued tobacco consumption will be integral for developing newer and more successful treatment options.

Neurobiology of nicotine use

Activation of nAChRs

Nicotine is recognized as the primary psychoactive and addictive element in tobacco, although other components of cigarette smoke such as monoamine oxidase inhibitors and the nicotine metabolites nornicotine and continine have psychoactive properties. When smoked, nicotine rapidly enters the bloodstream and reaches the brain in 10-20 seconds. The half-life of nicotine in plasma is about 2 hours, and metabolites are present in high concentrations for an extended period of time (Benowitz et al., 2009). Nicotine is an agonist for nicotinic acetylcholine receptors (nAChRs), which are ligand gated cation channels expressed throughout the central and peripheral nervous systems (Barik and Wonnacott, 2009). Nicotinic receptors, as a part of the cholinergic system, are particularly necessary for attentional processing (Poorthuis and Mansvelder, 2013; Sarter and Paolone, 2011). Nicotine and other nAChR agonists can enhance attention, working memory processes, and cognitive flexibility (Chan et al., 2007; Hahn et al., 2003), and are potential therapeutic targets for some psychiatric disorders (Poorthuis et al., 2009; Rezvani and Levin, 2001).

Nicotinic receptors are composed of a complex of subunits in either homomeric or heteromeric compositions. The differing composition of nicotinic receptor complexes result in variations in agonist affinity, receptor dynamics, and rates of desensitization (Feduccia et al., 2012). These receptors are located both pre-and post-synaptically, as well as on the soma of multiple types of neurons within the CNS (Penton and Lester, 2009). Based on the subunit composition of a nAChR, discrete receptor dynamics contribute to the influence of nicotinic receptor agonists (Brunzell and Picciotto, 2009) and contribute to the primary rewarding effects of nicotine. The distribution of these receptors in some circuits (including those relevant to nicotine addiction) are conserved across rodents and primates (Zoli et al., 2015).

Of particular importance in mediating the pharmacological effects of nicotine are homomeric receptors composed of α7 subunits, and heteromeric receptors containing β2 subunits, primarily α4β2 receptors (Couey et al., 2007; Penton and Lester, 2009; Perry et al., 1999). These two types of nAChR demonstrate different affinities for nicotine, and upon nicotine binding, show distinct rates of receptor desensitization and upregulation (Feduccia et al., 2012). Both chronic and acute exposure to nicotine influences the expression of nAChRs, resulting in upregulation in mesocorticolimbic areas such as the ventral tegmental area (VTA), prefrontal cortex (PFC), and striatum (Pistillo et al., 2016; Sarter and Paolone, 2011; Zoli et al., 2015). Nicotine exposure in both humans and rodents results in an increase in the expression of nAChRs throughout the brain (Marks et al., 2011; Moretti et al., 2010; Perry et al., 1999), and these receptors are necessary for mediating both the primary and reinforcement enhancing effects of nicotine (Palmatier et al., 2006).

Expression and role of nAChRs in mesocorticolimbic circuitry

The action of nicotine on nAChRs within mesocorticolimbic brain regions is of particular interest, as these regions are thought to mediate the reinforcing and motivational properties of drugs and drug-associated stimuli. This circuit includes the VTA, nucleus accumbens (NAc),

amygdala, and PFC. The VTA has been particularly well studied in terms of its necessity for mediating the effects of nicotine (Laviolette and van der Kooy, 2004). Dopaminergic projections that originate in the VTA are integral to the expression of the rewarding and reinforcing properties of nicotine (Balfour, 2015). Nicotinic cholinergic receptors are highly expressed within the VTA, on both dopaminergic and GABAergic neurons (Nashmi and Lester, 2006) as well as on presynaptic terminals of projections that originate in the PFC and NAc (Jones, 2004; Klink et al., 2001). The expression of nAChRs on multiple neuronal subtypes allows for finely tuned control of dopaminergic release throughout the brain. In addition, upon activation by nicotine, the manifold combinations of receptor subunits with differing affinity and receptor desensitization properties can produce an increase in activation of DA neurons that originate in the VTA (Markou, 2008; Wonnacott et al., 2006). Activation of nAChRs in the VTA can result in the release of dopamine in both the NAc and the PFC (Exley et al., 2013; Gotti et al., 2010; Livingstone et al., 2009; Nisell and Marcus, 1997; Perez et al., 2012), contributing to reward-associated behavior and the rewarding effects of nicotine. Agonism of nicotinic receptors within the VTA is not the only mediator of the primary reinforcing effects of nicotine, as glutamatergic, GABAergic, and opioid receptors have also been shown to modulate the effects of the drug (Kenny et al., 2009). Antagonism of mGluR5 receptors, or agonism of mGluR2/3 receptors (D'Souza and Markou, 2011; Liechti et al., 2007), μ opioid receptors, or GABAergic receptors (Corrigall et al., 2000) in the VTA can reduce self-administration of nicotine, suggesting that neurotransmitter systems, as well as activation of nicotinic receptors in the VTA, contribute to the primary reinforcing effects of nicotine.

The ventral striatum and PFC are important targets of dopaminergic projections from the VTA involved in the motivational properties of drugs and associated stimuli, as well as in executive control over behavior (Kesner and Churchwell, 2011; Nisell and Marcus, 1997; Singer et al., 2016). A small population of cholinergic interneurons, as well as nAChRs located presynaptically on dopaminergic terminals, influence firing of medium spiny neurons located

within the striatum (Berg et al., 2016; Pakhotin and Bracci, 2007; Zhou et al., 2002). In the PFC, the expression of nAChRs on pyramidal neurons themselves is debated, and this expression may be specific to certain cortical layers (Feduccia et al., 2012; Zoli et al., 2015). The receptors are expressed on terminals of glutamatergic and dopaminergic projection neurons, as well as GABAergic interneurons (Poorthuis et al., 2013). Nicotine influences plasticity in the PFC by increasing GABAergic transmission (Couey et al., 2007), resulting in decreased firing of pyramidal neurons, but also increases release of glutamate and dopamine from terminals that project into the PFC (Feduccia et al., 2012). The balance of receptor kinetics based on subunit composition on GABAergic and glutamatergic neurons within the PFC is thought to underlie the multifaceted effects of nicotine, from enhanced attentional processing to the detrimental effects of chronic nicotine exposure.

Reinforcing effects of nicotine

Nicotine has been identified as the primary pharmacological component responsible for tobacco addiction. The pharmacological activity of nicotine produces long lasting and measurable effects on reward and motivational systems of the brain. However, it has become clear that producing therapies solely targeted at disrupting the primary rewarding effects and alleviating withdrawal symptoms in dependent persons attempting to quit is not enough to produce long-term cessation (Prochaska and Benowitz, 2016). Specifically, the role of conditioned cues is vital for smoking maintenance, including the subjective feeling of craving that can lead to relapse (Childress et al., 1993; Rose, 2006).

Nicotine diverges from other drugs in that it has relatively weak primary reinforcing properties. For example, when given a choice between a cocaine- or nicotine-associated lever, rats will choose the cocaine-associated lever (Manzardo et al., 2002). Nicotine was originally notorious for the difficulty of achieving self-administration in rodent models, but robust self-administration can be achieved by the addition of environmental stimuli (Caggiula et al., 2001).

Environmental stimuli enhance self-administration of nicotine in clinical populations of smokers and in laboratory animals (Caggiula et al., 2001; Donny et al., 2003; Le Foll and Goldberg, 2006), and nicotine both self-administered and administered noncontingently increases responding for non-nicotine reinforcers such as a visual stimulus (Chaudhri et al., 2006; Donny et al., 2003; Palmatier et al., 2006). These results suggest that nicotine produces "reinforcement enhancing effects" in that it increases the incentive value of non-nicotine stimuli, simply by being administered or pharmacologically active during stimulus presentation (Palmatier et al., 2007).

These primary and reinforcement enhancing effects can be dissociated in both humans and animals. In one study, rats were shown to press a lever at similar rates for either nicotine infusion or presentation of a visual stimulus. Pairing the two outcomes increased responding for the combination of nicotine + stimulus, and receiving nicotine infusions increased lever responses for the visual stimulus alone (Palmatier et al., 2006). A similar effect has been demonstrated in human smokers given a choice between denicotinized cigarettes and IV nicotine, in that smokers chose the cigarettes and reported a reduction in withdrawal symptoms even though they did not provide the pharmacological effects of nicotine (Rose et al., 2010). These findings suggest that that the primary reinforcing properties of nicotine differ from other commonly abused drugs, which do not require secondary reinforcers to the same extent, and that nonpharmacological effects of nicotine contribute to its reinforcing effects. Thus, current treatment options that focus solely on nicotine replacement therapy may fall short as they only target the primary pharmacological effects of nicotine, and not the conditioned or behavioral effects.

Nicotine influence on cues and behavior

During the establishment of smoking behavior, neutral environmental and sensory stimuli are paired with the pharmacological effects of the drug, resulting in stimulus-outcome associations. In the absence of nicotine, these nicotine-associated stimuli can influence

subjective responses (Caggiula et al., 2001; Rose et al., 2010). Exposure to smoking-associated cues can increase the feeling of craving and urge to smoke (Ferguson and Shiffman, 2009). When denicotinized cigarettes are presented to smokers along with visual and olfactory smoking cues, participants report positive subjective experiences such as “liking” and “satisfaction”. These responses are similar to those reported with nicotine, and denicotinized cigarettes are able to reduce cigarette craving (Barrett, 2010; Brauer et al., 2001; Butschky et al., 1995; Gross et al., 1997). Obstructing the experience of these olfactory or visual smoking cues reduces the subjective effects of liking a cigarette, and also reduces smoking (Perkins et al., 2000). Craving for nicotine during withdrawal or abstinence can be associated with the lack of the pharmacological effects of the drug, and this outcome can be blunted with nicotine replacement therapies such as patches or nasal sprays. In humans using nicotine replacement therapy to reduce smoking, dermal nicotine patches can reduce craving caused by nicotine withdrawal, without reducing craving induced by exposure to nicotine-associated environmental stimuli (Ferguson and Shiffman, 2009; Tiffany et al., 2000; Waters et al., 2004). This suggests that it may be necessary to combine therapies that target the pharmacological properties of the drug with therapies that disrupt the strong associations formed between – and amplified by – the drug and conditioned cues (Prochaska and Benowitz, 2016; Rose, 2006; Rose and Levin, 1991).

Studying individual variability in animal models of addiction

When studying drug abuse, it is important to note that only a subset of those who initiate drug use recreationally will transition to substance abuse or addiction. Behavioral models that take into account individual variability are particularly useful for understanding and predicting characteristics that are associated with addiction vulnerability. Traits that emerge in humans and suggest a link to addiction, either because they are likely to occur in populations that are at risk of developing a substance abuse disorder, or because they occur in populations that are already dependent, can often be modeled in animals and used to probe the underlying

behavioral, neurobiological, and even genetic causes. The use of animal models allows investigators to conduct controlled experiments and evaluate these factors individually. Use of animal models allows for longitudinal studies and within subject designs, and the assessment of potential predictive relationships between behavior and the effect of a drug. It allows for the identification of translational risk factors that can be used to measure these traits as they relate to the different stages of addiction that occur in humans; mainly initiation, escalation, maintenance, extinction, and relapse or reinstatement (Carroll et al., 2009). Behaviors linked to addiction vulnerability aren't only measures of drug use themselves, they are often behaviors that can be tested in the absence of drug exposure. The processes that mediate the expression of these behaviors are thought to overlap with circuitry that is dysfunctional in drug addicted populations. These traits are often linked to deficits in cognitive or behavioral control, stress and anxiety, risk taking, and reward processing (Carroll et al., 2009; Sinha, 2011).

Behaviors used to study addiction vulnerability

Several behaviors are commonly linked to addiction vulnerability in humans and in animals. One of the earlier traits identified in animals was the "sensation-seeking" phenotype in which animals that exhibited novelty seeking and higher locomotor activity in a novel environment were also more likely to show elevated amphetamine self-administration (Piazza et al., 1989). Animals that display this phenotype are also more likely to self-administer cocaine (Belin et al., 2011; Belin and Deroche-Gamonet, 2012), morphine (Pelloux et al., 2006) and alcohol (Nadal et al., 2002). Sensation-seeking is a phenotype that has been identified in human smokers as well, as they are more likely to score highly on measures of response to novelty and novelty-seeking (Carton et al., 1994; Malmberg et al., 2013; Redolat et al., 2009), although the difference in novelty seeking between dependent and nondependent smokers is less pronounced (Harmsen et al., 2006). In animal models of sensation seeking that assess nicotine addiction vulnerability, the connection to this behavior is also less established than for other

drugs of abuse (Falco and Bevins, 2015). Studies that differentiate between high and low sensation-seeking rodents to predict locomotor sensitization to nicotine find inconsistent results (Falco and Bevins, 2015), suggesting that this model may be good for predicting addiction vulnerability in some drugs of abuse, but may not be optimal for predicting vulnerability to nicotine addiction.

Individual differences in impulsivity are also linked to addiction vulnerability for multiple drugs, including nicotine. Impulsivity presents as a disruption of behavioral control, either in inhibitory control of actions or suboptimal choice decisions, and is thought to be linked to deficits in cortical control of behavior (Crews and Boettiger, 2009). Drug exposure can result in neuroadaptations that promote impulsivity (Taylor and Jentsch, 2001), and expression of impulsivity has been shown to predict drug self-administration (Dalley et al., 2008). Impulsivity has been consistently identified in populations of smokers, indicating that smokers are more likely to be impulsive both when measured using behavioral questionnaires (Billieux et al., 2007; Doran et al., 2009; Spillane et al., 2010), and based on performance on delay discounting tasks (Mitchell, 1999). In animal models of impulsivity, rats that show high impulsive choice as measured by delay discounting tasks or high impulsive action as measured by the 5-choice serial reaction time task (5CSRTT) also reach higher breakpoints on a progressive ratio schedule for nicotine self- administration. These animals were resistant to extinction, displayed more cue-induced reinstatement, and high impulsivity was related to elevated dopamine release in the accumbens and medial prefrontal cortex (Diergaarde et al., 2017, 2011). Measuring impulsivity is a useful way to link behavior to general addiction vulnerability, but there is still a need for additional models of behavioral dysfunction that can predict nicotine dependence.

Sex differences in behavior and drug abuse

In addition to cognitive and behavioral differences that can contribute to addiction vulnerability, biological differences in sex can also interact with other components that

contribute to the likelihood of developing of a substance use disorder and relapse to drug-seeking. When comparing sexes in both humans and animals, some studies have indicated that levels of circulating gonadal hormones can contribute to individual behaviors and may influence both smoking and cessation in females. One report comparing menstrual cycle phase and estradiol or progesterone in female smokers found that the ratio of estradiol to progesterone predicted smoking behavior (Schiller et al., 2012). In a study of smokers administering IV nicotine, a difference in nicotine self-administration in females arose based on menstrual cycle phase. In addition, participants described experiencing fewer of the subjective effects of nicotine during the luteal phase and exhibited better cognition during the follicular phase (Devito et al., 2013). In women attempting to abstain from smoking, a difference in outcome has been noted based on the phase of the menstrual cycle in which she quits (Allen et al., 2008; Carpenter et al., 2008; Weinberger et al., 2015) and increased progesterone levels are associated with more successful quit attempts (Saladin et al., 2015).

In tasks that relate to individual differences in behavioral or cognitive control, sex differences can arise in terms of sensation-seeking and impulsivity. Male and female smokers both show increased sensation-seeking compared to nonsmokers, and females can potentially rate higher on this trait than males (Carton et al., 1994). In a study that attempted to measure sensitivity to the reinforcing effects of nicotine upon initial experience of nicotine administration in nonsmokers, Perkins (2008) reported multiple sex differences in behavioral traits such as impulsivity and novelty-seeking, and the evaluation of subjective and reinforcing effects of nicotine. Potential sex differences have been found in some studies of impulsive action (Fields et al., 2009; Reynolds et al., 2007). In studies of impulsive choice using delay discounting tasks, males and females show variability in the relative likelihood of making impulsive choices (Heyman and Gibb, 2006; Reynolds et al., 2004). This suggests that while there are some potential sex differences in behavioral measures, both in smokers and nonsmokers, there are

also numerous inconsistencies and the extent of these sex effects vary across studies in humans.

Females can also differ from males in the response to smoking-related cues. In a study in which male and female smokers were exposed to cues that predicted cigarette availability, females reported stronger craving after cue presentation, as well as an increased physiological response of salivation after cue presentation compared to males (Field and Duka, 2004). Sex differences in responses to cues may exist during early adulthood, as presentation of smoking cues can elicit a more craving in young adult female smokers than males (Carpenter et al., 2014). Sex differences in cue reactivity can also be measured by fMRI in brain regions that respond to smoking cues (McClernon et al., 2008).

Some sex differences also emerge in animal models of individual variability and drug-associated behavior. In studies that test impulsive action using the 5CSRTT, males and females vary depending on the particular measures of this trait being assessed. Bayless (2012) found that females made fewer premature responses on the task, suggesting increased inhibitory control compared to males, while males demonstrated more attention on the task as they made fewer omissions. In animals bred to exhibit high or low levels of impulsivity, sex differences emerge in cocaine self-administration and cocaine primed reinstatement (Perry et al., 2008). Female mice exposed to nicotine show decreased sensitivity to increasing concentrations of nicotine during self-administration and increased conditioned place preference for nicotine, but do not differ from males in locomotor sensitization to nicotine (Isiegas et al., 2009). On a neurobiological level, sex differences can exist in the dopaminergic system during development due to the organizational effects of gonadal hormones (Becker, 1999; Connell et al., 2004), and differences in dopamine receptors in multiple areas of the brain critical to reward and motivation (Andersen et al., 2000) leading to an increase in dopaminergic transmission in females. Jentsch and Taylor (2003) found opposing changes in females and males when they were intact or gonadectomized, indicating a potential contribution of circulating hormones in both male and

female rats to the expression of impulsivity. On a whole, these studies indicate that sex can interact with other behavioral measures to produce differences in the extent of responses as well as differences in the degree of an effect of drugs such as nicotine.

Pavlovian conditioned approach behavior

Development of sign-and goal-tracking behaviors

In classically conditioned or Pavlovian learning, the consistent presentation of a conditioned stimulus (CS) is paired with the response-noncontingent delivery of a reward (unconditioned stimulus, US). The association between the CS and US is a predictive relationship, but in some individuals the CS itself can become valuable and modify behavior. This CS, whether it is associated with drugs of abuse or a natural reward, can influence emotional and motivational states (Cardinal et al., 2002). It can inspire behavior such as approach to the CS, even though that behavior is not explicitly rewarded (Robinson and Berridge, 2003). Brown and Jenkins (1968) demonstrated this effect in a classic study with pigeons, in which presentation of a key light as a CS was paired with the delivery of a food US. After repeated pairings of the key light and food delivery, the pigeons approached and pecked at the key light. The pigeons were not required to perform this behavior to receive their reward, but the conditioned response to CS presentation developed regardless.

Since this seminal study of Pavlovian conditioning, much has been learned about the expression of this behavior, the neurocircuitry that underlies it, and how it can serve as a predictor of addiction vulnerability. These CS-US associations form in humans as well as animals, and are a necessary and adaptive form of learning. This learning can become maladaptive, however, as these associations also form between drugs of abuse and environmental stimuli that are associated with the effects of the drug. Understanding these learned associations, in terms of their formation, strength, and ability to influence behavior, is crucial for the study and treatment of drug abuse and addiction (Saunders and Robinson, 2013).

In animals, the Pavlovian conditioned approach or “autoshaping” behavioral model is particularly helpful to measure these stimulus-outcome associations. This model allows for researchers to study the formation of these associations, and attempt to extinguish or diminish their ability to inspire unhealthy or maladaptive behavior. The behavior can be used to assess the associations between both drug-associated and nondrug stimuli, how drugs of abuse can influence the formation of these associations, and why some individuals may be predisposed to develop stronger conditioned associations than others (Flagel and Robinson, 2017; Peters and De Vries, 2014).

Modeling Pavlovian approach behavior also allows for the study of the incentive motivational properties of conditioned cues. Stimuli that have been linked to a reward, drug or non-drug, can develop incentive properties. These stimuli acquire salience, are able to inspire behavior, and influence an organism’s emotional or motivational state apart from their conditioned association with the US (Cardinal et al., 2002). Incentive stimuli exhibit certain measurable characteristics in animals, in that they are able to attract attention and stimulate approach toward the CS, act as conditioned reinforcers, and motivate behavior and reward seeking (Flagel et al., 2009). This process is helpful and adaptive in most situations, as it can encourage an animal to approach and interact with salient stimuli that are associated with natural reward such as food, water, or sex (Burns and Domjan, 2001, 1996; Jenkins and Moore, 1973). However, the attribution of incentive salience can become maladaptive in the case of drug use, as drugs are thought to specifically usurp the circuitry involved in the formation of these associations, and in the case of addiction, sensitizing them to the point that they can become detrimental the organism (Flagel and Robinson, 2017). Thus, studying the formation of Pavlovian associations, as well as the individual differences that contribute to the enhanced attribution of salience to the conditioned cue, is of particular interest in drug abuse research.

In humans, we can see the importance of Pavlovian conditioned associations for drug-associated cues in studies of attentional bias and cue reactivity. Smokers exhibit increased

physiological and neuronal activity to smoking cues (Childress et al., 1993; Field and Duka, 2004; Waters et al., 2004) and this activity can be related to craving and treatment success (Janes et al., 2010; Tiffany et al., 2000; Waters et al., 2004). Attentional bias to drug associated cues is expressed in people who show heavy use of multiple drugs, including nicotine (Field et al., 2009; Townshend and Duka, 2001). Smokers are quicker to respond to smoking-related cues compared to neutral cues (Bradley et al., 2004; Chanon et al., 2010; Mogg et al., 2003) and are more likely to be distracted by these stimuli in situations in which allocating excessive attention to smoking cues is detrimental to task performance (Waters et al., 2003). This behavior is thought to reflect the enhanced salience of the drug-associated cues, suggesting the recruitment of underlying incentive motivational circuitry when processing and reacting to stimulus presentation.

Thus, for situations in humans and animals where the conditioned cue is able to influence behavior in its own right, it is thought to have developed incentive properties in addition to predicting the US. Depending on the parameters of the Pavlovian task, two distinct conditioned responses can emerge. Sign tracking, in which an animal approaches and interacts with the CS, or goal tracking, where upon CS presentation the animal approaches the location of US delivery (Flagel et al., 2009). The likelihood that these responses will emerge depends on the characteristics of the CS, as a localizable light or lever stimulus that is spatially separated from the location of US delivery is needed to produce and measure the sign tracking conditioned response, and auditory cues are appropriate for producing solely the goal tracking response (Meyer et al., 2014).

Measurements of goal tracking, or approach to the US, are suitable for gauging the development of Pavlovian associations and attempting to manipulate the memory or strength of this predictive relationship. This can be attempted by extinguishing the conditioned responding behavior generated by CS presentation, and then testing the reinstatement of the response after presentation of the conditioned cue. In paradigms where both conditioned responses emerge,

individual differences can arise in the propensity of an animal to exhibit either approach response. Sign tracking has been of particular interest for its relation to addiction vulnerability. When animals display the sign tracking CR, it is thought that the CS itself has acquired incentive salient properties. The CS is able to motivate approach and behavior toward it, act as a conditioned reinforcer, and inspire Pavlovian to instrumental transfer. Cues that elicit approach are not necessarily incentive stimuli, but they do still represent the predictive CS-US relationship (Flagel et al., 2009; Robinson et al., 2014).

In paradigms that allow for the expression of sign tracking, previous drug exposure or the presentation of drug-associated cues can motivate approach. Multiple drugs of abuse have been shown to motivate sign tracking if the drug is provided as the US. Although one study demonstrated that a cocaine US does not promote sign tracking (Kearns and Weiss, 2004), others have been successful (Saunders and Robinson, 2011; Uslaner et al., 2006). In addition, sign tracking occurs if sweetened or unsweetened alcohol (Krank et al., 2008; Tomie et al., 1998) or heroin and other opiate receptor agonists (Peters and De Vries, 2014; Yager et al., 2014) are used as the US. These results demonstrate that salience can be attributed to a cue that specifically predicts drug administration. Additional experiments are needed, however, to directly compare the extent to which an animal will sign track to a CS that predicts a drug or a separate CS that predicts a food reward. In addition, the ability of a nicotine US to specifically promote sign tracking has yet to be established.

Sign-and goal-tracking conditioned responses demonstrate different characteristics that are linked to individual addiction vulnerability. The sign tracking response is resistant to extinction (Ahrens et al., 2016; Peters and De Vries, 2014), and prone to spontaneous recovery (Palmatier et al., 2013; Peters and De Vries, 2014). Use of conditioned taste aversion or outcome devaluation can reduce expression of goal tracking, but not sign tracking (Morrison et al., 2015). Sign tracking is linked to other behaviors that are thought to confer addiction vulnerability such as impulsivity, as rats that show higher impulsive choice on a delay

discounting task acquire the sign tracking response faster, and are more likely to sign track than low impulsive rats (Tomie et al., 1998). Sign trackers also show increased psychomotor sensitization to cocaine exposure compared to goal trackers (Flagel et al., 2007). Physiological changes exist between sign and goal trackers, as autoshaping sessions that produce sign tracking result in elevation of plasma corticosterone in rats. This increase is present after the first session of CS-US pairings, even before developing the sign tracking conditioned response, suggesting that Pavlovian conditioning in itself generates arousal. This arousal may continue to a greater extent in sign tracking animals than in animals that don't develop this response (Tomie et al., 2004, 2002). Additional differences emerge between sign and goal trackers on a neuronal and biochemical level (Tomie et al., 2008), suggesting altered developmental and neurobiological factors that contribute to the behavior.

Brain circuitry involved in Pavlovian conditioned approach

A number of different brain regions have been proposed to be responsible for the expression of conditioned approach to a Pavlovian CS. These cue-motivated behaviors are thought to activate a broad network of reward and motivational circuitry in both humans and animals (Cardinal et al., 2002; Kalivas and Volkow, 2005). In attempting to understand the circuitry that contributes to these behaviors, many studies have been able to distinguish between regions that are required to represent the predictive value of the CS, and those that a required to represent the incentive value of the CS. Pavlovian conditioned approach allows differentiation between these two related processes by investigating both sign tracking and goal tracking animals and identifying circuitry that is specifically involved in the separate populations.

One key component of the circuit required for both the acquisition and expression of Pavlovian approach behaviors is the dopaminergic projection from the VTA to the NAc. Phasic dopamine (DA) released into the NAc has been shown to shift from the US to the CS after the formation of a relationship between the two, and it is thought that this dopaminergic innervation

can represent the learned value of the CS (Day et al., 2007; Schultz et al., 2015). In animals trained to express Pavlovian approach behavior, phasic DA released in to the NAc core shifts to the CS after learning in sign trackers but not goal trackers, suggesting that DA represents the incentive properties of the reward cue, and not just the predictive relationship (Flagel et al., 2011). Additional studies suggest the importance of DA in the NAc specifically for sign tracking, as injection of the nonspecific dopamine receptor antagonist flupenthixol into the NAc core impairs sign tracking, but not goal tracking (Saunders and Robinson, 2012). This impairment was visible on the first trial of Pavlovian conditioning sessions, suggesting that antagonism of dopaminergic receptors is not causing a gradual decrease in behavior, similar to extinction learning. Animals that have received flupenthixol into the NAc will still orient to the cue when it is presented, but they will not approach (Yager et al., 2014), indicating that antagonism of DA specifically depresses the approach response that signals acquired incentive motivational value.

Others have continued to demonstrate that DA in the NAc is integral to the sign tracking response, as injection with the antipsychotics haloperidol and olanzapine both preferentially reduce sign tracking (Danna and Elmer, 2010). Sign tracking animals express more D1 dopamine receptor mRNA in the NAc than goal trackers, and after 5 days of conditioned approach training, goal trackers show an increase in the expression of mRNA for tyrosine hydroxylase, the dopamine transporter, and D2 receptors, suggesting that separate adaptations within the dopaminergic system occur in animals that display separate phenotypes (Flagel et al., 2007). In a study where mice were trained on a Pavlovian task to approach the US, knocking out NMDA receptors on dopaminergic neurons did not reduce goal tracking behavior even though there was a decrease in DA released into the accumbens, further suggesting the preferential involvement of DA release in the NAc for eliciting the sign tracking response, but not goal tracking (Parker et al., 2010).

In addition to relevant dopaminergic innervation of the NAc for the expression of sign tracking, the striatum as a whole is also implicated in this circuit for assigning motivational

salience to the conditioned cue and motivating the approach response. Neurons in the NAc core encode the presentation of the CS and the expression of approach to the CS (Day et al., 2006). The dorsal striatum has been implicated in behavioral responding, as agonism of dopaminergic or μ-opioid receptors in the dorsolateral striatum can boost the preferred sign or goal tracking responses in individual animals, enhancing the already elevated motivational salience of the CS or US (DiFeliceantonio and Berridge, 2016). The NAc also receives input from the PFC, and cortico-striatal projections are implicated in both drug abuse and the expression of behaviors that confer addiction vulnerability (Everitt et al., 2008; Jentsch and Pennington, 2014).

Additional regions of interest in the circuit involved in producing Pavlovian conditioned approach are the amygdala, both central and basolateral nuclei, and the PFC. Experiments examining the basolateral (BLA) and central amygdala (CeA) show inconsistent involvement in the expression of Pavlovian approach behaviors. For example, some studies have indicated that lesions of the CeA do not influence the acquisition or expression of sign tracking, but lesions of the BLA do impair the behavior (Chang et al., 2012a, 2012b). Other studies show the opposite effect, in that the CeA is required for the acquisition and expression autoshaping, but lesions of the BLA do not affect the behavior (Everitt et al., 1999; Parkinson et al., 2000).

Multiple regions of the PFC may contribute significantly to the acquisition and expression of Pavlovian approach behaviors, particularly the representation of the CS-US relationship and the attribution of incentive motivational properties to the conditioned cue. Several regions of the PFC have been identified as specifically active in sign tracking animals, based on activation of immediate early genes after behavioral training (Flagel et al., 2011; Yager et al., 2014). Lesion studies have identified the anterior cingulate and, to some extent, the medial prefrontal cortex as required for the acquisition and expression of approach to the CS (Bussey et al., 1997). Monoaminergic transmission in the anterior cingulate has also been identified as a potential contributor to the acquisition and expression of autoshaping behavior, as animals that have acquired CS-US associations show increased expression of both norepinephrine and serotonin

in this region (Tomie et al., 2004). The orbitofrontal cortex is necessary for the acquisition of goal tracking behaviors (Chudasama and Robbins, 2003; Ostlund and Balleine, 2007), but it's requirement for sign tracking behaviors has not been directly established. Inconsistencies in the literature concerning the involvement of specific regions suggests that there is still much to be discovered about the full circuit that contributes to this behavior, and the individual contributions of each region.

Nicotine effects on Pavlovian conditioned approach

In experiments that specifically focus on nicotine exposure, multiple effects of the drug have been demonstrated on Pavlovian approach, the acquired salience of the conditioned cue, and the influence of neurotransmitter or receptor systems in the brain. In studies of Pavlovian discriminative approach behavior, in which water-deprived rats were trained with a localizable tone+light compound stimulus and water US, rats that were exposed to nicotine for several days prior to training or that received injections of nicotine after each training session showed enhanced approach to the US receptacle (Olausson et al., 2003). In a separate study utilizing the same Pavlovian conditioning procedures, rats were trained in the absence of nicotine, and then tested for the ability of nicotine to enhance conditioned reinforcement. In this task, two novel levers were inserted into the chamber, and responding on the active lever presented the conditioned CS from Pavlovian training. Nicotine exposure increased responding for the conditioned reinforcer, and treatment with the α4β2 receptor antagonist, mecamylamine, blocked this enhancement by nicotine (Olausson et al., 2004). This result suggests that nicotine can influence the incentive properties of conditioned stimuli, even if it was not present for the initial formation of the stimulus-outcome association. These studies indicate that nicotine can enhance approach to the US, as well as increase the incentive motivational properties of the conditioned cue even if sign tracking is not measured.

Guy and Fletcher (2013) conducted a series of experiments that replicated and extended those of Olausson and colleagues. In these experiments, water deprived rats were also trained in a Pavlovian conditioned approach procedure that utilized a light+tone CS and a water US. Nicotine was administered immediately before each conditioning session and enhanced conditioned approach to the US receptacle compared to controls. Operant responding for the conditioned reinforcer was tested in the presence or absence of nicotine in animals that had received saline during training, and in those that had received nicotine. Nicotine only enhanced responding for the conditioned reinforcer in animals that had received nicotine during Pavlovian training, in contrast to the results seen by Olausson (2004). Additionally, systemic injections with mecamylamine, or the α7 antagonist DhβE, blocked the enhancement of responding by nicotine. A subsequent study from this group replicated the Pavlovian conditioned approach results, beginning nicotine exposure at different times during training (Guy and Fletcher, 2014a). A lever CS was also added to measure the sign tracking response, and this study found that nicotine enhanced sign tracking but not goal tracking. In tests for conditioned reinforcement of the previously trained lever CS, nicotine only enhanced responding for the conditioned reinforcer if it was injected before the test. D1 and D2 receptor antagonists, as well as a 5-HT2C receptor agonist, attenuated responding on the test of conditioned reinforcement in both nicotine-exposed and control animals, suggesting that these receptors are also involved in the expression of responding, regardless of the presence of nicotine (Guy and Fletcher, 2014b).

Palmatier and colleagues (2013) characterized Pavlovian conditioned responding in rats trained to approach an illuminated receptacle as a CS and receive a sucrose reward in either the same, or a spatially separated receptacle. Nicotine did not increase conditioned approach when the CS and US receptacle were the same, but upon spatial separation, nicotine-exposed animals performed more of the sign-and goal-tracking conditioned responses than controls did. While conditioned responses were extinguished during extinction training, both nicotine-exposed and control rats exhibited spontaneous recovery, and nicotine animals reinstated

approach behavior more than controls. Additionally, when nicotine injections were withheld, the sign tracking conditioned response persisted for 24 days of training without nicotine, suggesting that the effect of nicotine had produced long lasting changes to behavior. In an additional study using a similar paradigm and the same dose of nicotine, dopaminergic antagonists were administered to manipulate conditioned approach in nicotine- or saline-exposed animals. Antagonists for D1 and D2/3 receptors, as well as a non-specific dopaminergic antagonist, reduced conditioned approach in both groups, and specifically reduced goal tracking in controls and sign tracking in nicotine-exposed rats. This result points to the importance of the dopaminergic system for the expression of conditioned approach, and particularly in mediating the ability of nicotine to enhance the incentive motivational properties of a conditioned cue (Palmatier et al., 2014).

Additional studies have used Pavlovian conditioned approach training to pre-classify animals as sign trackers or goal trackers and then assessed additional behavioral responses in the presence of nicotine. In one study, sign- and goal-tracking rats self-administered nicotine (IV), and then were tested in extinction and during cue-induced reinstatement. Sign trackers showed increased lever pressing for nicotine during self-administration and enhanced cue-induced reinstatement, but no difference in extinction. Additionally, nicotine was given during Pavlovian approach training in a separate group of animals, and nicotine was shown to enhance sign tracking but not goal tracking (Versaggi et al., 2016). A separate study that used similar Pavlovian conditioned approach training to classify rats as sign or goal trackers subsequently measured the extent to which a cue paired with IV nicotine acquired the properties of an incentive stimulus. The nicotine paired cue was equally attractive to sign and goal trackers, an effect that differs from results of other studies with cocaine- and opiate-paired cues. The nicotine-paired cue was a more effective conditioned reinforcer in sign trackers, but only at the highest dose of nicotine tested (Yager and Robinson, 2015).

Together, these recent studies indicate the reinforcement- and incentive-amplifying effects of nicotine by demonstrating that nicotine exposure can increase both sign-and goal-tracking conditioned responses. In paradigms that measure both behaviors concurrently, nicotine appears to preferentially enhance sign tracking. The experiments that demonstrate nicotine enhancement of goal tracking (Guy and Fletcher, 2013; Olausson et al., 2004, 2003), even in the presence of a visual stimulus, did not measure approach to the CS, and it is possible that sign tracking was also occurring in these studies. Most studies indicate that nicotine must be pharmacologically active at the time of stimulus presentation to heighten the incentive properties of a conditioned cue, although Olausson (2004) suggested that this may not always be the case. It appears that the incentive amplifying effects of nicotine separate it from other drugs of abuse (Yager and Robinson, 2015). Thus, continued evaluation of the effects of nicotine, particularly in paradigms that assess Pavlovian learning, could contribute to our understanding of nicotine's influence on conditioned cues.

Involvement of the orbitofrontal cortex in appetitive approach behavior

The OFC has been extensively evaluated across multiple behavioral paradigms and using numerous techniques in humans, nonhuman primates, and other mammals. This has resulted in a multitude of experimental findings, at times with inconsistent or even opposing results, that propose several functions for the OFC. The lack of a unifying and agreed upon function of the OFC is a tangible problem in the literature, one that many labs are attempting to rectify (Dom et al., 2005; Stalnaker et al., 2015; Wilson et al., 2014). Of interest for this dissertation is the potential involvement of the OFC in both humans and animals in representing stimulus-outcome associations and behavioral flexibility.

The OFC has been shown to be required for tasks that rely on Pavlovian stimulus-outcome associations, including conditioned approach. In an autoshaping task in which animals approached a CS+, lesions of the OFC disrupted the behavior when animals were lesioned before training (i.e. acquisition) but not when lesions were made after training (Chudasama and

Robbins, 2003). In a separate study in which the OFC was lesioned after Pavlovian approach training, lesions of the OFC did not affect the ability of the rats to acquire a conditioned taste aversion for the previously trained US, but the lesions prevented them from modifying their conditioned approach response during a subsequent test session (Gallagher et al., 1999). Additionally, in rats trained to associate two different auditory CS with separate rewards, those with lesions of the lateral and ventrolateral OFC continued to show appropriate conditioned approach to the goal cups. After the contingency between one CS-US pair was degraded, animals reduced responding to both the degraded and nondegraded stimulus (Ostlund and Balleine, 2007). These lesion studies show that the OFC is needed to acquire a conditioned approach response, but may not be necessary to express the behavior once it is learned. However, changing either the value of the expected outcome or the predictive relationship between the CS and US results in performance deficits in OFC lesioned animals. As demonstrated by these studies using Pavlovian approach tasks, the OFC also seems to be involved not only in the acquisition of the conditioned approach behavior, but in updating behavior based on new learning and inhibiting previously learned behaviors.

Additional studies support the finding that the loss of OFC function after learning a task does not prevent expression of the behavior, but does influence updating of behavior after new learning. During reversal tasks in which the OFC is lesioned or inactivated, animals were impaired in the ability to develop a new behavior, but were able to inhibit the previously learned response (Burke et al., 2009; Keiflin et al., 2013). Another study found that inactivating the OFC did not prevent learning within the session, but animals did not show a consistent change in behavior across multiple sessions (Panayi and Killcross, 2014). These studies suggest that the OFC is needed to update cue-outcome contingencies and to guide future behavior based on this newly learned association.

The OFC has been studied in the context of behavioral inhibition using tasks that measure impulsive actions and impulsive choices. Neurons in the OFC fire during response

inhibition in a go/no go task (Schoenbaum et al., 1998; Tremblay and Schultz, 1999), and OFC lesions increase perseverative errors on a 5CSRTT task (Chudasama et al., 2003). Firing patterns of OFC neurons during delay discounting tasks suggest that they signal the extent of the delay, and are correlated with impending discounting behavior (Roesch et al., 2006a, 2006b). Interestingly, lesion and inactivation studies have shown that the loss of OFC function either increases impulsive choice on a delay discounting task (Mobini et al 2003, Rudebeck 2006) or decreases impulsive choice (Kehramin et al., 2002; Winstanley et al., 2004). It has been proposed that this discrepancy stems from either heterogeneity in populations of OFC neurons that are being targeted, the extent of OFC lesions that may encompass both lateral and medial regions, or the presence of cues that signal the length of a delay within the task (Mar et al., 2011; Zeeb et al., 2010).

Studies of the OFC in populations of humans, either those with damage to the OFC from trauma or in those with substance abuse disorders, have indicated similar results to those from animal models (Dom et al., 2005; Volkow and Fowler, 2000). Humans with damage to the OFC are more likely to show increased impulsivity when measured by impulsive choice or on delay discounting tasks (Sellitto et al., 2010). Humans with OFC damage also show impaired performance on reversal tasks, and an inability to correctly and quickly update behavior based on new learning (Berlin et al., 2004). The OFC is active during both the anticipation and receipt of a reward (O'Doherty et al., 2002), and the amygdala and OFC appear to show similar activation in response to a reward-predictive cue and after reward devaluation (Gottfried et al., 2003). Human studies that differentiate between the lateral OFC and medial OFC indicate separate functions of these regions, similar to the dissociation of function indicated in animal studies (discussed below). The medial and lateral OFC may differentially represent expected rewards and detrimental outcomes or punishment (O'Doherty et al., 2001), and multiple other studies have indicated functional specificity of subregions of the OFC (Noonan et al., 2012).

Research conducted in populations of smokers and nonsmokers indicates dysfunctional activation of the OFC, particularly in response to smoking related cues or during craving (McClernon et al., 2008). Multiple studies using imaging techniques have indicated that smokers show increased activation of prefrontal regions, including the OFC, when presented with smoking-related cues compared to neutral cues (Brody et al., 2002; Claus et al., 2013; Kang et al., 2012). This increased activation is also correlated to the experience of craving, either cue-induced craving (Brody et al., 2002; Franklin et al., 2007; Kang et al., 2012) or abstinence-induced craving (Wang et al., 2007). In addition to cue-induced activation and activity correlated with craving, the OFC is also dysfunctional at multiple stages of nicotine use including abstinence or withdrawal (Goldstein, 2002). This dysfunction, such as reduced general activity of the OFC, and abnormal activation during task performance, persists during long term abstinence over multiple years (Neuhaus et al., 2006). Differentiation between the function of OFC subregions is also emerging in studies of smoking related activation, as the mOFC has been identified as specifically activated during cue-induced craving, and activation in this region can be blunted by manipulation with transcranial magnetic stimulation (Hayashi et al., 2013) or the pharmacological smoking cessation drug varenicline (Franklin et al., 2011).

Orbitofrontal circuitry

The OFC is part of the circuit that contributes to incentive salience of conditioned cues and their ability to motivate behavior. The OFC sends a glutamatergic projection to the VTA, and receives a dopaminergic projection from the VTA. These two regions are involved in encoding predicted outcomes and learning from unexpected outcomes, as neuronal firing in the OFC that signals anticipated rewards is correlated with firing of dopaminergic neurons in the VTA that encode prediction errors (Takahashi et al., 2009). Lesions of the OFC result in the failure of dopaminergic neurons to show normal error signaling in response to omitted or unexpected rewards and disconnection of the OFC and VTA by ipsilateral inactivation of either structure also disrupts learning from unexpected outcomes (Takahashi et al., 2011, 2009). This

suggests that the OFC and VTA are cooperating to encode outcome expectancies, and to facilitate new learning after unexpected or unpredicted outcomes.

Tracing studies have reported variable results about the density of projections from the OFC to the NAc core and shell (Morecraft et al., 1992; Ongür and Price, 2000; Schilman et al., 2008), yet numerous studies have reported the importance of functional connectivity between these regions. In a study that investigated the ability of the OFC and NAc core to encode a cue that predicts a reward of a specific magnitude, lesions of the OFC left the NAc capable of firing in response to the cue, but neurons in the NAc no longer encoded the magnitude of the predicted outcome (Cooch et al., 2015). This result suggests that the OFC and NAc are at least functionally connected and that this connectivity is required to encode the value of an expected reward. Concurrent inhibition of neuronal activation in the OFC, combined with enhanced activation of the NAc core and shell resulted in impairment on tasks that required behavioral inhibition (Meyer and Bucci, 2016), further suggesting that functional connectivity between the ventral striatum and OFC contributes to behavior. Additionally, projections between the OFC and dorsal striatum have been shown to be responsible for encoding goal-directed or habitual actions. Gremel and Costa (2013) recorded neuronal firing from neurons in the OFC, dorsolateral (DLS), and dorsomedial (DMS) striatum during a task that allowed for a within session switch from goal-directed to habitual response strategies, and found that the OFC and DMS were more engaged in goal-directed actions. This finding indicates that the OFC is involved in representing the value of an action, separate from representing stimulus-outcome associations.

The strong reciprocal projections between the OFC and the BLA have been implicated in behavior and encoding outcome value. In a study that performed electrophysiological recordings from the OFC of rats with lesions to the BLA after a reversal of the previously learned cue-outcome associations, fewer OFC neurons fired selectively in response to cue presentation or in anticipation of the expected outcome, but OFC neurons still fired based on the identity of the

outcome (Schoenbaum et al., 2003). Several other studies have implicated the OFC and BLA as being functionally connected during reversals, and encoding the new learning associated with the reversal task. It is thought that within this OFC and BLA circuit, both regions encode cue-outcome associations, but the BLA specifically updates the associations based on new learning which the OFC integrates with previously learned associations. This forms a representation of the current task state that can be accessed by the BLA and other regions of the brain (Sharpe and Schoenbaum, 2016; Wilson et al., 2014).

Of particular importance when studying the function of the OFC is to differentiate between subregions. Multiple studies have indicated that the OFC is not a homogeneous structure, as it contains at least two distinct subregions and heterogeneous populations of projection neurons. It is likely that many of the inconsistencies in the reported functions of the OFC come from the lack of regional specificity when manipulating or measuring this region. In particular, many of the lesion studies that purport to find functions of the OFC eliminate both the medial and lateral orbitofrontal cortex, which may have diverse and potentially opposing functions. For example, one study that attempted to rectify conflicting results of the OFC's role in impulsivity in rat studies using delay discounting suggested that when either the whole OFC or lOFC was lesioned, animals showed the expected increase in impulsivity, and chose the smaller reward at shorter delay times than controls. When only the mOFC was lesioned, animals displayed a decrease in impulsive choice by choosing the larger, delayed reward. Animals also showed contrasting behaviors based on the region of the OFC that was lesioned when faced with a reversal task (Mar et al., 2011). Additional studies of the mOFC have corroborated this result in rodents, such as the finding that the mOFC is sensitive to the value of an outcome after tests of outcome devaluation or a use of a progressive ratio schedule of reinforcement, indicating that inhibition of the mOFC reduced representation of the value of an outcome (Gourley et al., 2016). Additional studies in humans have indicated a dissociation from the medial OFC and vmPFC, and the lateral OFC as well (Noonan et al., 2012). These studies

indicate the need for additional research that establishes the specific functions of subregions of the OFC leading to a more nuanced understanding of the function of the OFC as a whole.

Summary of aims

The studies described here, along with multiple others, demonstrate that nicotine is capable of influencing Pavlovian approach behaviors by enhancing the incentive properties of a Pavlovian conditioned cue. Numerous studies also indicate that the OFC is involved in encoding stimulus-outcome associations and should be active in tasks of Pavlovian conditioned approach behavior. The OFC appears to be responsible for updating these associations to incorporate new learning, but other regions of the circuit that are functionally connected to the OFC also share in this function. The exact role of the OFC has yet to be confirmed, including the involvement of this region in specifically encoding the incentive value of a CS, and conditioned approach behavior. Thus, in this dissertation, we endeavored to investigate the influence of the OFC on conditioned approach, and measure the influence of nicotine on the expression of these behaviors. In addition, we considered the possibility that nicotine influences male and female rats differently, by influencing both conditioned approach behavior as well as protein expression in brain regions crucial for the expression of conditioned responding.

We hypothesize that nicotine exposure will enhance Pavlovian approach behaviors in both males and females, and reduce the flexibility of these behaviors after a change in outcome value. In addition, we hypothesize that this behavior will be encoded in the OFC. Specifically, the OFC will encode the incentive value of the reward-predictive cue, as well as encoding approach behaviors, and nicotine will blunt the ability of the OFC to control conditioned responses. Finally, we hypothesize animals exposed to nicotine, and potentially animals that sign track, will show reduced protein expression in the OFC.

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Chapter 2: Orbitofrontal participation in sign- and goal-tracking conditioned responses: Effects of nicotine[1]

Introduction

Environmental stimuli associated with nicotine or other drugs of abuse can acquire incentive motivational properties, becoming salient, attractive, and able to motivate behavior (Robinson and Berridge, 1993). In humans attempting to abstain from drug use, encountering these 'incentives' - stimuli that acquire motivational properties based on associations with drug rewards (Logan, 1964) - can lead to craving and promote relapse (Obrien et al., 1992). Bio-behavioral models of substance dependence implicate long-term changes in the brain circuitry that mediates responses to incentives as central to substance use disorders (Di Chiara et al., 1992; Robinson and Berridge, 1993). Preclinical studies have confirmed that frontolimbic circuitry plays a critical role in the motivational effects of many drugs of abuse (Kalivas and Volkow, 2005). This circuit includes ascending dopaminergic projections from the midbrain, including the ventral tegmental area, and descending glutamatergic projections from the frontal cortex, including the anterior cingulate gyrus and prefrontal cortex (PFC). These projections converge on subcortical circuits that include the ventral striatum, ventral pallidum, and subthalamic nucleus.

The PFC has been implicated in substance dependence because of its role in top-down control of behavior, attention, decision making, and other functions that, when compromised, contribute to addiction vulnerability (Perry et al., 2010). Chronic drug use increases the influence of ascending midbrain systems while reducing cognitive control, resulting in an

[1] Stringfield, S.J., Palmatier, M.I., Boettiger, C.A., Robinson, D.L., 2017. Orbitofrontal participation in sign- and goal-tracking conditioned responses: Effects of nicotine. Neuropharmacology 116, 208–223. doi:10.1016/j.neuropharm.2016.12.020

enhanced drive to seek the drug and a decrease in the ability to inhibit drug-seeking (Olausson et al., 2007). The orbitofrontal cortex (OFC), in particular, has been linked to incentive motivation and representations of outcome value or salience in both humans and animals (Gottfried et al., 2003b; Ogawa et al., 2013), as well as the expression of behavioral responses and reward-seeking behaviors (Burton et al., 2014; Moorman and Aston-Jones, 2014). While the exact function of the OFC has yet to be precisely defined (see Stalnaker et al., 2015 for review) the OFC has consistently been characterized as involved in behaviors such as impulsivity (Mar et al., 2011; Zeeb et al., 2010) and Pavlovian conditioned approach (Chudasama and Robbins, 2003; Gallagher et al., 1999; Ostlund and Balleine, 2007).

Incentive stimuli that predict both drug and non-drug rewards evoke 'Pavlovian conditioned approach' behavior which can take one of two forms. Approach behaviors oriented toward the location of reward delivery are traditionally referred to as 'goal tracking,' whereas behaviors oriented toward the location of the incentive, if it is spatially separated from the reward, are referred to as 'sign tracking' (Brown and Jenkins, 1968). Sign tracking has recently come under increasing scrutiny in substance dependence research because of its association with drug abuse vulnerability (Saunders and Robinson, 2013; Tomie et al., 2008). Although both sign and goal tracking rely on the same mesotelencephalic systems implicated in substance dependence (Flagel et al., 2011b; Saunders and Robinson, 2012), individual subjects who display a greater propensity to sign track show increased drug self-administration (Saunders and Robinson, 2011; Versaggi et al., 2016). These individual differences are also linked to variation in stress responses, neurotransmitter release, and neuronal activation in areas including the PFC and the nucleus accumbens (Saunders and Robinson, 2013; Tomie et al., 2008). For example, one study found that c-fos mRNA induction in the OFC was increased only in animals that displayed the sign-tracking response (Flagel et al., 2011a). While it appears that the OFC is involved in Pavlovian conditioned behaviors, there is still much to be learned, including the differential involvement of this region based on specific conditioned responses.

Recent studies from multiple laboratories suggest a special relationship between the effects of nicotine and approach to incentives (Palmatier et al., 2014; Versaggi et al., 2016; Yager and Robinson, 2015). The interaction between nicotine and incentives is especially relevant to tobacco use and dependence because preclinical studies have repeatedly demonstrated that nicotine is a weak primary reinforcer (Foll and Goldberg, 2009; Palmatier et al., 2006). Caggiula, Donny, Chaudhri and others (Caggiula et al. 2001; Donny et al. 2003; Chaudhri et al. 2006) have argued that nicotine self-administration follows from three effects of nicotine on behavior. First, nicotine is a primary reinforcer, albeit a weak one, meaning that nicotine delivery alone supports self-administration. Second, nicotine is a reinforcement enhancer; i.e., nicotine delivery increases responding for non-drug reinforcers (Chaudhri et al. 2007; Donny et al. 2003; Palmatier et al. 2006). Third, serving as a primary reinforcer, nicotine can establish associated non-drug stimuli as 'conditioned reinforcers' (i.e., incentives; Palmatier et al. 2008). More recently, Palmatier and colleagues (Palmatier et al., 2014, 2013a, 2012) have argued that the second effect of nicotine, enhanced responding for non-drug reinforcers, reflects an effect of nicotine on underlying neurobiological substrates that mediate responses to incentives, including conditioned stimuli. Accordingly, they have found that nicotine promotes Pavlovian conditioned approach, including sign-tracking (Palmatier et al., 2013b), and that the increase in approach is abolished by dopaminergic antagonists (Palmatier et al., 2014).

The present study sought to more thoroughly explore the neurobiological underpinnings of the incentive-promoting effects of nicotine by evaluating the role of the OFC in sign-and goal-tracking. We hypothesized that the OFC would be directly involved in both sign- and goal-tracking conditioned responses, and that nicotine exposure would reduce the ability of the OFC to exert top down control over this behavior. We tested this hypothesis with pharmacological inactivation of the OFC and by examining OFC firing patterns *in vivo* during Pavlovian conditioning sessions.

Methods

Animals

Adult, male, Sprague Dawley rats (225-250g on arrival) were purchased from Harlan/ Envigo (Indianapolis, IN), pair housed during initial training, and then individually housed after surgery. 16 animals were used for Experiment 1, and 25 animals were used for Experiment 2. Animals were provided with food and water *ad libitum* during the entire experiment. Rats were housed in a vivarium on a 12:12 hour light:dark cycle, and all experiments were conducted during the light cycle. All procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Behavioral training and nicotine regimen

Before training, animals were allowed 1-hour access to the 20% sucrose (w/v) solution that would be used as the unconditioned stimulus. Animals were then assigned to either a nicotine exposure group (NIC) or a saline control group (SAL). Nicotine hydrogen tartrate salt (Sigma-Aldrich, St. Louis, MO) was dissolved in sterile saline and the pH was adjusted to 7.0 ± 0.2. Animals in the NIC group received one injection of 0.4mg/kg nicotine (s.c., calculated using the freebase form) and animals in the SAL group received an equivalent volume of saline for two days prior to conditioning to habituate them to the injection procedure. This dose was chosen because it is commonly used for repeated subcutaneous injections of nicotine, and we and others have previously shown that this dose influences conditioned responding (e.g., Guy and Fletcher, 2014; Palmatier et al., 2013b). Training sessions were conducted in standard behavioral chambers (MedAssociates, St Albans, VT) assembled with Plexiglas walls. A recessed reward receptacle, stimulus light, and retractable lever directly below the light were located on one wall of the chamber, and a house light was positioned on the opposite wall. A photobeam detector across the reward cup detected head entries into the receptacle. Animals were habituated to the testing chambers during one day of receptacle training, in which they

were injected with the assigned drug or control solution, returned to their home cage for 10 min, and then placed in the testing chamber for 5 min before session initiation. During this session, 20% sucrose was dispensed into the receptacle on a variable interval (VI) 120 s schedule. Animals rarely failed to consume the reward, and NIC and SAL groups did not differ in the amount of fluid left in the reward cups at the end of the session (data not shown). Next, 20 (Experiment 1) or 25 (Experiment 2) Pavlovian conditioning sessions were conducted, Monday-Friday, in which the animals were injected with nicotine or saline 15 min before session initiation as described above. The house light was illuminated throughout the session and stimulus-reward pairings occurred on a VI 120 s reinforcement schedule. The conditioned stimulus (cue) consisted of illumination of a cue light and extension of the lever located directly below the light. Cue presentations lasted 30s, and were immediately followed by 0.1ml of 20% sucrose dispensed into the reward receptacle. Each session consisted of 15 cue-reward trials. After these training sessions, all animals were habituated to custom-built Plexiglas chambers inside sound attenuated boxes which had similar components but were optimized for electrophysiology recordings (Fanelli et al., 2013), for an additional 5 days before surgery.

Surgery

For Experiment 1, rats were anesthetized with isoflurane and implanted bilaterally with 26-gauge stainless steel cannulae (Plastics One, Roanoke, VA) aimed at the lateral OFC (3.7mm anterior, 2.7mm lateral, 4.0mm ventral from bregma). For Experiment 2, rats were implanted with two microwire electrode arrays (NB Labs, Denison, TX), each consisting of 8 stainless-steel, Teflon-coated wires that were 50-µm in diameter and spaced 0.5mm apart in a 2x4 configuration. Fixed-placement arrays were used as we planned to record behavior over several days and conditions, and we aimed to sample the same population of neurons (if not the same individual neurons) across each recording day. Arrays were placed bilaterally into the OFC, (centered at 3.7mm anterior, 2.7mm lateral from bregma, 5.0mm ventral from the adjacent

skull surface). For both experiments, animals were allowed to recover from surgery for at least 7 days before resuming Pavlovian conditioning sessions.

Experiment 1: Intracranial microinfusions

After recovery, rats underwent 3-5 days of Pavlovian conditioning sessions to ensure that behavior remained constant. Two days before the intracranial microinfusions, animals were habituated to the procedure immediately before a standard Pavlovian conditioning session. Rats were held by the experimenter and 33-gauge stainless steel injection cannulae (Plastics One) that protruded 1mm beyond the guide cannulae were inserted and left in place for 4 min, mimicking the subsequent infusion procedure. On the test day, animals were infused with a cocktail of the $GABA_A$ receptor agonist muscimol and the $GABA_B$ receptor agonist baclofen (Sigma-Aldrich, 0.125µg of each drug/0.5µl) or saline vehicle. These doses were chosen because they have previously been shown to affect OFC-dependent behavior (Zeeb et al., 2010) without influencing locomotor activity (St. Onge and Floresco, 2010). Injection cannulae were left in place for 1 min before and 1 min after the infusion to ensure accurate diffusion of the drug. Each infusion occurred over 2 min, during which 0.5µl of drug cocktail or vehicle was infused into each hemisphere at a rate of 0.25µl/min. Next, animals were immediately injected with the previously assigned solution of either nicotine or saline and Pavlovian conditioning sessions occurred as described above.

Experiment 2: In vivo electrophysiology

Rats underwent 2-3 Pavlovian conditioning sessions after surgery to confirm that behavior remained consistent before being habituated to a flexible tether connected to the headstage assembly and electrode arrays. After habituation, electrophysiological recordings were conducted during Pavlovian conditioning sessions and test sessions. Electrophysiological recordings were conducted as previously described (Fanelli et al., 2013; Robinson and Carelli, 2008) using a multichannel acquisition processor (MAP system using SortClient software; Plexon Inc, Dallas TX) to record neuronal activity. Briefly, animals were tethered and placed in

the recording chamber for 15 min before the start of the session. During this time, a differential reference was manually selected for each electrode array and thresholds were set for all microwires. Pavlovian conditioning sessions were conducted as described above and timestamps from MedAssociates software registering within-session events (cue onset, lever press, receptacle entry, reward delivery) were aligned with neuronal activity recorded with the MAP system. After each session, cell sorting was conducted using Plexon Offline Sorter software. Timestamp data were imported and further analyzed using Neuroexplorer (NEX Technologies, Madison AL) and custom-written programs in MATLAB (Mathworks, Natick, MA).

Histology

Animals were injected with 1.5 g/kg urethane solution (Sigma-Aldrich, 50% w/w in saline, i.p.). Once anesthetized, a 10µA current was passed through each stainless-steel microwire in rats from Experiment 2 to leave an iron deposit at the location of the electrode tip. All rats were perfused with 10% formaldehyde solution. The brain was removed and fixed in 30% sucrose cryoprotectant before being frozen, 40-µm sections were then taken on a cryostat. Sections were stained with potassium ferracyanide and thionin to visualize individual electrode or cannula placements. Cannula (Figure 1A) and electrode (Figure 1B) placements were marked based on sections from the Paxinos and Watson rat brain atlas (Paxinos and Watson, 1998).

Behavioral data analysis and statistics

Data from Pavlovian conditioning sessions were collected with MedAssociates software and compiled using custom-written programs in R (R Core Team, version 3.1.2). Lever deflections and latencies to enter the receptacle or press the lever were averaged across trials for each animal. As the reward receptacle was present throughout the entire session, receptacle *elevation scores* (Palmatier et al., 2013b) were used to assess enhanced receptacle entries that occurred as a result of cue presentation. Elevation scores were calculated by subtracting the number of receptacle entries that occurred 30 s immediately before each cue presentation (pre-cue period) from the number of receptacle entries that occurred during each 30 s cue

presentation (cue period). Thus, a positive elevation score indicates that an animal increased this response specifically during cue presentation. Lever and receptacle probability scores were calculated by taking the number of trials during which an animal pressed the lever or entered the receptacle at least once, and dividing by 15 total trials. Comparisons of behavioral responses during training and on test days were conducted using 2-way repeated-measures ANOVA in Sigma Plot (Systat Software Inc, San Jose CA) followed by Tukey's HSD post-hoc comparisons when appropriate. Probability scores were analyzed with the genmod procedure in SAS (SAS Institute Inc, Cary, NC) using a Wald's chi-square within the context of a logistic regression model with effects for each drug treatment by week or test day combination and standard error adjusted for multiple observations within rats. Behavioral responses were also compared between NIC and SAL groups on the final day of training (Experiment 1: Day 20, Experiment 2: Day 25) by independent samples t-test or Mann-Whitney U-test, depending on the distribution of the data.

Cell firing data analysis and statistics

Baseline firing rates of cells for each group were calculated by averaging the spike rates during the 60s immediately before house light illumination that signaled session initiation. Firing rates of individual neurons surrounding within-session events were normalized by dividing the firing rate at the event of interest by the mean firing rate over the whole session. For population analysis, the normalized activity of all cells for each treatment or behavior group was aligned to the event of interest and smoothed with a moving average of 250 ms in 50 ms steps. Statistical analysis of population activity was completed using a Mann-Whitney rank-sum test or Kruskal-Wallis one way ANOVA on ranks in Sigma Plot software.

To identify changes in firing patterns that are not apparent with population analysis, individual neurons that demonstrated phasic activity by significantly changing their firing rate around within-session events were classified using z-scores. For cue onset, firing rates during a 2 s pre-target window (baseline) were compared to firing during a 500ms target window

immediately after the cue was presented. For all other events, a change in firing during a target window 500ms before and after the event occurred was compared to a 2 s baseline period. Z-scores were calculated based on the magnitude of the change in firing rate, and cells with a z-score between -2 and 2 were classified as non-phasic. A z-score of >2 was classified as excitatory activity, and a z-score less than -2 was classified as inhibitory activity.

Results

Animals were excluded from Experiment 1 if one or both cannula were not placed in the OFC. A total of 16 rats underwent surgery and 2 animals were excluded from the study based on incorrect cannula placements. Placement of cannula tips for remaining animals are depicted in Figure 1A, both the NIC and SAL groups included 7 animals. Individual units recorded in Experiment 2 were excluded if the corresponding wire was not placed in the OFC. Animals were excluded if no cells remained on wires that were correctly placed in the OFC. A total of 25 animals underwent surgery and 1 animal was removed based on incorrect electrode placements. Placements of remaining electrodes are depicted in Figure 1B. One animal was removed from analysis for failing to complete the saline test day. A total of 12 animals in the SAL group and 11 animals in the NIC group were included in the study.

Experiment 1: Inactivation of the OFC

To assess differences in acquisition of Pavlovian conditioned approach behavior, NIC and SAL animals were compared over 20 days of training. Measures of conditioned responding are collapsed across 4 weeks and means ± SEM are presented in Figure 2. Over time, both experimental groups developed conditioned responses to cue presentation, approaching both the lever and reward receptacle. Rats in the NIC group were more likely to exhibit sign tracking behaviors than controls. Both groups decreased the latency to approach the reward receptacle and increased both the number of lever presses per trial and the probability of pressing the lever over 4 weeks of training. NIC rats, relative to SAL rats, showed a non-significant decrease in lever latency over 4 weeks of training [Fig 2A, $F(1,12) = 4.3$, $p=0.061$], and a statistically

significant decrease on the final day of training [$t(12) = 2.3$, $p<0.05$]. NIC rats exhibited more lever pressing than SAL rats over the last two weeks of training [Fig 2B, group × week interaction $F(3,36) = 13.1$, $p<0.001$], which was also visible on the final day of training [$t(12) = 3.8$, $p<0.01$]. During acquisition, both groups of animals were similarly likely to approach and press the lever at least once per trial [Fig 2C, $X^2(4) = 6.2$, $p>0.05$], but a difference in lever probability emerged by the final day of acquisition [$t(12) = 2.3$, $p<0.05$]. There were no differences between groups or any interactions for the goal tracking measures of receptacle latency, receptacle elevation score, or receptacle probability (Figs 2D-F).

While we hypothesize that nicotine will increase the likelihood that an animal will show sign tracking behaviors, we anticipated that significant individual variability would arise within treatment groups. Individual rats can develop sign- and goal-tracking behaviors regardless of drug treatment, and this individual variability emerged during training. To illustrate this, we plotted the behavior of each individual rat in the NIC and SAL groups on the last day of training (Figs 2A-F, right). NIC animals displayed more sign tracking behavior on average, but individuals within both drug exposure groups revealed a diverse behavioral profile, including both sign- and goal-tracking conditioned responses.

Pharmacological inactivation of the OFC

To assess the involvement of the OFC in mediating the sign- and goal-tracking conditioned responses and the ability of nicotine exposure to influence OFC control of these behaviors, animals received intra-OFC infusions of the GABA receptor agonists baclofen and muscimol. OFC inactivation reduced conditioned responding in both NIC and SAL animals compared to vehicle infusion, but did not completely abolish behavior (Figure 3). In fact, there were selective changes in some behaviors, and no difference in responding in others. Both NIC and SAL groups showed a reduction in sign-tracking behaviors after OFC inactivation, compared to vehicle infusion. Group differences that were present after vehicle infusion remained after inactivation. There was an increase in lever latency after inactivation (Fig 3A),

with a main effect of infusion [$F(1,12) = 8.6$, $p<0.05$] and a main effect of group [$F(1,12) = 7.3$, $p<0.05$]. Lever presses per trial (Fig 3B) were reduced in both groups after inactivation [$F(1,12) = 4.9$ $p<0.05$] while group differences remained [$F(1,12) = 5.8$, $p<0.05$]. The same held for lever probability (Fig 3C) with main effects of infusion [$X^2(1) = 7.3$, $p<0.01$] and group [$X^2(2) = 6.6$, $p<0.05$]. The reduction but not complete loss of sign-tracking conditioned responses suggests that the OFC influences the expression of this behavior.

There were fewer effects of inactivation on goal-tracking behaviors. For receptacle elevation score (Fig 3E) only NIC animals reduced their elevation score after inactivation of the OFC, as demonstrated by a group × infusion interaction [$F(1,12) = 6.0$ $p<0.05$]. There was no difference in latency to approach the reward cup (Fig 3D), or probability of performing a receptacle entry after GABA agonist infusion compared to control conditions (Fig 3F). Thus, while both NIC and SAL animals reduced their sign tracking behaviors after inactivation of the OFC, there were few changes to goal tracking behaviors, suggesting that the change in behavior was not due to gross deficits in locomotor activity. While measures of receptacle latency and probability of entering the receptacle did not change after OFC inactivation in either group, NIC animals did show a decrease in receptacle elevation score while SAL animals were unchanged.

Experiment 2 – Single-unit recording from the OFC during Pavlovian conditioning

A second cohort of rats was trained with the purpose of conducting *in vivo* electrophysiology during Pavlovian conditioning sessions. This group of animals trained as described for Experiment 1, but training occurred over 25 days and behavioral data were collapsed across 5 weeks (Fig 4). Additionally, we include comparisons between treatment groups on the last day of training and plot individual behavioral responses on that day (Fig 4A-F, right). In this cohort, NIC animals displayed increased sign tracking and goal tracking behaviors. On measures of sign tracking, NIC animals decreased their lever latency during acquisition [Fig 4A, group × week interaction $F(4,92) = 38.6$, $p<0.05$ and showed an increase in lever probability

[Fig 4C, group × week interaction $X^2(4) = 13.3$ $p<0.05$] Post-hoc tests indicate that NIC animals were significantly faster to approach and more likely to press the lever during the last 4 weeks of training. On the last day of training (Day 25), group differences between NIC and SAL animals did not reach significance for lever latency [MWU=42.0,p=0.053], but NIC rats demonstrated a higher probability of lever pressing on this day [MWU=28, $p<0.01$]. There was no difference in lever presses per trial between NIC and SAL animals, although the mean lever presses for NIC animals was consistently higher than SAL animals each week. On the last day of training, the difference between NIC and SAL groups in number of lever presses did not reach statistical significance [Fig 4B, MWU=44.5, $p=0.072$]. The lack of a difference on this measure can be attributed to the high variability in SAL animals, and the existence of high-pressing SAL animals. NIC animals also showed increases in goal tracking behaviors. NIC animals were generally faster to and more likely to approach the receptacle, as demonstrated by main effects of group for receptacle latency [Fig 4D, $F(1,23) = 4.4$, $p<0.05$] and receptacle probability [Fig 4F, $X^2(5) = 11.9$, $p<0.05$]. For receptacle elevation score, NIC animals showed a higher elevation score over the last 4 weeks of training [Fig 4E, $F(4,92) = 3.5$, $p<0.05$]. On the last day of training, NIC and SAL groups did not differ in terms of latency to press the lever [Fig 4D, MWU=59.0, $p>0.05$]. However, animals in the NIC group showed a higher receptacle elevation score [Fig 4E, $t(23)=3.3$, $p<0.01$] and a greater probability of pressing the lever [Fig 4F, MWU=24.5, $p<0.01$].

Single-unit recording from the OFC

We next performed *in vivo* single unit recordings of OFC neurons during a standard Pavlovian conditioning session. We analyzed 153 neurons from 12 SAL animals and 120 neurons from 11 NIC animals. 2 animals from the NIC group were removed from analysis for not completing the saline test day, or due to incorrect electrode placements. Basal firing rates in NIC and SAL rats were analyzed immediately before session initiation. No significant differences in basal firing rate arose between groups (mean SAL firing rate: 4.7 ± 0.25 mean

NIC firing rate: 5.2 ± 0.3 Hz, MWU statistic = 7972.5, p = 0.231). There was also no difference in average firing rate across the whole session (mean SAL firing rate: 4.6 ± 0.23 Hz, mean NIC firing rate: 5.2 ± 0.29 Hz, MWU statistic =7744.0, p=0.118).

Example raster plots and peristimulus time histograms for a single cell from one NIC animal are presented in Figure 5, depicting firing patterns centered on behavioral responses and within-session events. This cell did not exhibit a change in firing rate during a receptacle entry prior to cue presentation (Fig 5A) but showed an increase in firing rate when the animal performed a goal tracking conditioned response (Fig 5B) and during reward retrieval (Fig 5C). There was no change in firing rate when the animal pressed the lever (Fig 5D), but the cell exhibited changes in firing rate at cue presentation (Fig 5E), and during the first second after cue offset (Fig 5F). Similar firing patterns were visible when population activity was analyzed.

Mean population activity for NIC and SAL groups for those same events are presented in Figures 6(A) and 7(A-D). Figure 6A depicts peristimulus time histograms centered at cue onset and cue offset for NIC and SAL groups. These events are both predictors of reward, as cue onset inspires measurable conditioned responding, and cue offset is a more proximal predictor of reward availability. Firing in both NIC and SAL animals increased in response to cue onset and in response to cue offset. Cells from NIC animals increased their firing rate less than cells from SAL animals did at cue onset. Analysis of the peak firing rate during the 1s after cue onset by Kruskal-Wallis one way ANOVA on ranks indicates a difference between NIC and SAL groups (Fig 6A, left; $H(1) = 9.1$, $p<0.01$). There was no difference in firing rate during the 1s after cue offset (Fig 6A, right). It is possible that the observed changes in OFC neuronal firing were due to alterations in the testing environment, and therefore not specific to the presentation of reward-predictive stimuli. To test this possibility, we analyzed population activity of OFC cells to an additional stimulus: the house light illumination that marked the start of the conditioning session (Supplemental figure S1). We found no increase in OFC firing rate upon illumination of the house light.

Next we examined individual neuronal firing patterns at cue onset and offset. Figures 6B and 6C depict mean firing rates for phasically active neurons at cue onset and offset for SAL (Fig 6B) and NIC (Fig 6C) groups. Phasically active neurons are classified as those cells that significantly increased or decreased their firing rate compared to a 2s baseline (blue shaded area) prior to the time of the stimulus. Phasic cells from SAL and NIC animals displayed a primarily excitatory response at cue onset and cue offset, for cells from SAL animals, 24% were excited and 5% were inhibited at cue onset, while 23% were excited and 5% were inhibited at cue offset. The same pattern was seen in cells from NIC rats, where 18% of cells were excited and 4% were inhibited at cue onset, and 19% were excited and 6% were inhibited at cue offset.

In addition to changes in firing rates due to cue presentation, we analyzed OFC neuronal firing during conditioned responses in the same Pavlovian conditioning session. Events of interest were the first receptacle entries in the 30 s before cue onset, during each trial, or immediately after cue offset, as well as the first lever press of each trial. Population and phasic activity were analyzed and compared for NIC and SAL groups. OFC neurons showed an increase in firing rate particularly during receptacle entries that occur as part of a goal tracking conditioned response and when retrieving the reward after the cue presentation, but not during receptacle entries that occurred before cue presentation (Fig 7). Population activity centered on receptacle entries that occur in the absence of the cue indicate no change in mean firing rate during this behavior. Analysis of phasic activity in both NIC and SAL animals indicates that a proportion of neurons are excited surrounding this behavior, with 20% and 6% of SAL cells and 23% and 6% of NIC cells being excited or inhibited, respectively (Fig 7A). During the first receptacle entry that occurred during cue presentation, OFC neurons exhibited a pronounced increase in firing rate. The peak firing rate for SAL cells was significantly higher than that of NIC cells (Kruskal-Wallis $H(1) = 5.7$, $p<0.05$). In addition, 33% of SAL units and 29% of NIC units were phasically excited, while 8% and 11% were inhibited (Fig 7B). No peak differences arose between NIC and SAL cells during the first receptacle entry immediately after cue offset, when

the animal retrieves the reward. The highest proportion of phasically excited cells for each group was present during this period, with 41% of SAL cells and 34% of NIC cells being excited, and 12% of SAL cells and 11% of NIC cells being inhibited (Fig 7C). Finally, when the first lever press of each trial was analyzed, we found that there was no increase in population firing rate surrounding this action. This can be explained by the roughly equal proportion of cells that were excited and inhibited, with 17% and 13% of SAL cells being excited or inhibited, and 19% and 17% of NIC cells showing excitation or inhibition surrounding the event (Fig 7D). Thus, the increase in peak firing and proportion of phasically active cells during a cue-evoked conditioned response compared to a general receptacle entry suggests that the OFC encodes these actions differently. In comparison, there was a much less distinct change in population activity during a lever press, although OFC neurons were both phasically excited and inhibited during this behavior.

Pavlovian conditioned approach behavior in the absence of nicotine

We next injected NIC animals with saline instead of nicotine before testing to investigate whether the observed effects on Pavlovian conditioned responding were due to acute exposure to the drug or to lasting effects of repeated nicotine exposure. SAL animals received a saline injection as always, and served as a control for the reproducibility of this behavior. On the saline test day (Figure 8), NIC animals exhibited less conditioned responding on both sign- and goal-tracking measures while SAL animals did not change their behavior across the two days. There was a main effect of test day for lever presses [Fig 8B, $F(1,21) = 8.5$, $p<0.01$], which is explained by high variability in two animals in the SAL group that drastically reduced their lever pressing from the baseline day to the test day, while all other SAL animals remained within ± 2.4 lever presses per trial between the two days. There was a group × test day interaction for lever latency [Fig 8A, $F(1,21) = 8.4$, $p<0.01$], lever press probability [Fig 8C, X^2 (1) = 6.3, $p<0.05$], and receptacle elevation score [Fig 8E, $F(1,21) = 6.7$, $p<0.05$]. Post-hoc comparisons indicate that NIC animals displayed more of these behaviors on the baseline day and reduced their

conditioned behaviors on the saline test day while SAL animals showed no change. These results suggest that the acute effect of nicotine is responsible for the enhanced conditioned responding, as NIC animals reduced their behavior to SAL levels in the absence the drug.

Phasic cell firing in the absence of nicotine

In addition to behavior, we also measured neuronal firing during the saline test day. Analysis of basal firing rates recorded prior to session initiation for SAL animals did not yield any statistically significant differences (mean baseline day: 4.7 ±0.25 Hz, mean saline test day: 4.2 ±0.26 Hz, MWU statistic = 8201.0, p= 0.197) nor were there any differences in whole session firing rate (mean baseline day: 4.6 ±0.23 Hz, mean saline test day: 4.1 ±0.23 Hz, MWU statistic = 8286.0, p=0.247). There was a difference in basal firing rate in NIC animals on the regular Pavlovian session compared to the saline test session (mean baseline day: 5.2 ±0.3 Hz, mean saline test day: 4.0 ±0.27Hz, MWU statistic = 5226.5, $p<0.01$) and in whole session firing rate (mean baseline day: 5.2 ± 0.29 Hz, mean saline test day: 3.9 ±0.25 Hz, MWU statistic = 5048.0, $p<0.01$). However, basal firing rates and whole session firing rates did not differ between NIC and SAL animals on either the baseline day or the saline test day.

Peak normalized firing rates to within-session events were compared for each exposure group on the saline test day and baseline day. There were no statistically significant differences for SAL animals across the two test days on any measure (Figure 9A). For cells from NIC animals, there was an increase in peak firing at cue onset and [Kruskal-Wallis $H(1) = 7.5$, $p<0.01$] and at cue offset [Kruskal- Wallis $H(1) = 4.8$, $p<0.05$] after injection with saline instead of nicotine (Figure 9B). When peak firing rates for NIC and SAL animals were compared on the saline test day, there was no difference between them (Figure 9C). Thus, withholding the nicotine injection resulted in a reduction in conditioned responding and an increase in OFC neuronal firing to within-session events.

Discussion

In two cohorts of animals, we found that nicotine increased conditioned responding; sign-tracking behaviors were elevated in both Experiments 1 and 2 while goal-tracking behaviors were increased in Experiment 2. Inactivation of the OFC primarily reduced sign tracking and additionally reduced goal tracking in NIC rats. Electrophysiological recordings indicated that the OFC is active in response to the onset of a reward-predictive cue and during the retrieval of the reward. Goal tracking was encoded in about 30% of OFC neurons via phasic excitations that were time-locked to the behavior. In contrast, less than 20% of OFC neurons exhibited phasic excitations to sign tracking behaviors, which was not sufficient to produce a change in population activity. Chronic treatment with nicotine blunted the increase in OFC population firing rate at cue onset and this reduction in firing was recovered when nicotine treatment was discontinued, suggesting that nicotine acutely reduces phasic firing of OFC neurons. Nicotine-induced enhancement of conditioned approach also declined to control levels when nicotine treatments were discontinued, suggesting that the changes in firing rate observed in the OFC may play a role in nicotine-enhanced conditioned approach. However, this association cannot be explicitly discerned from the inactivation study and should be validated empirically.

Our model produced sign- and goal-tracking behavior similar to previous reports (Flagel et al., 2009; Palmatier et al., 2013b; Versaggi et al., 2016), as control animals exhibited individual preferences for the sign or goal tracking behavior. In addition, nicotine exposure increased the likelihood that an animal would display a sign tracking response in Experiments 1 and 2. This enhancement in approach to the conditioned cue in NIC animals fits with previous accounts of nicotine's ability to enhance the incentive value of a conditioned cue, even one that is not particularly associated with delivery of nicotine itself (Chaudhri et al., 2006; Palmatier et al., 2013b; Yager and Robinson, 2015). We see that nicotine increased goal tracking in Experiment 2 but not Experiment 1, possibly because of the smaller number of animals in

Experiment 1. Many studies that observe populations of animals that sign and goal track include much larger cohorts of animals to achieve the full range of behavior (Meyer et al., 2012). Previous work has also pointed to the effect of colony and vendor differences on the behavioral traits of animals (Fitzpatrick et al., 2013), although all animals from this study were obtained from the same vendor and location. We have previously reported enhanced goal tracking in NIC-exposed animals (Palmatier et al., 2013b), similar to the Experiment 2 results reported here. This interesting result prompts further investigation into the nature of nicotine's incentive amplifying effects, as other drugs of abuse, such as cocaine and alcohol, have been shown to preferentially enhance sign tracking (Krank et al., 2008; McClory and Spear, 2014; Uslaner et al., 2006). Nicotine is capable of increasing conditioned approach in animals pre-classified as sign and goal trackers, but on tests of conditioned reinforcement, nicotine enhances conditioned reinforcement in sign tracking animals specifically (Yager and Robinson, 2015). This suggests that while nicotine can enhance both conditioned responses, drug exposure may impact animals differently based on individual predispositions.

We aimed to discern the function and involvement of the OFC in both sign- and goal-tracking components of Pavlovian conditioned approach. Previous studies involving permanent lesions of the OFC indicated involvement of this region in approach to the location of reward delivery and noted deficits in behavior when updating stimulus-outcome associations and representations of the value of the cue or reward (e.g., Ostlund & Balleine 2007; Chudasama & Robbins 2003b). Here, we show that inactivation of the OFC by infusion of $GABA_A$ and $GABA_B$ receptor agonists (Experiment 1) reduced expression of sign tracking regardless of nicotine exposure. Yet, when neuronal firing rates were analyzed surrounding a lever press (Experiment 2), we saw little phasic firing of OFC cells. In one study (Flagel et al., 2011a), OFC activation was measured by c-fos mRNA expression when the cue was presented without reward after 3 days of extinction to the context, and only sign-tracking animals displayed an increase in OFC c-fos expression. Our pharmacological inactivation experiment agrees with this study, in that there

was a reduction in sign tracking after OFC inactivation. This result, tempered by the lack of population activity or robust phasic firing during expression of the behavior suggests that the OFC is involved in promoting the conditioned response, but that the behavior itself is not explicitly encoded in the firing rate of OFC neurons. The OFC is part of a much broader circuit that stimulates the sign-tracking response, particularly in terms of mesocorticolimbic circuitry that includes the nucleus accumbens (Cooch et al., 2015) and is required for the attribution of incentive salience to a cue (Flagel et al., 2011b). The function of the OFC during sign tracking may be to represent the association between the cue and expected outcome and encode the anticipatory state evoked by cue presentation, allowing other components of the circuit to access this representation and invoke the actual behavioral response (Gallagher et al., 1999). Recent descriptions of the function of the OFC, which suggest that it serves to integrate a multitude of cortical, subcortical, and sensory inputs to create a representation of the current task state (Wilson et al., 2014), may provide an explanation for the role of the OFC during sign-tracking.

Conversely, inactivation (Experiment 1) produced a limited reduction in goal tracking that only occurred in NIC animals. Physiologically (Experiment 2), there was a time-locked excitation of OFC neurons during cue-evoked receptacle entries as well as during retrieval of the reward, but not during receptacle entries that occurred in the absence of the cue. This suggests that neurons in the OFC are specifically encoding receptacle entries associated with anticipation of the expected outcome, which aligns with previous reports of single-unit activity in the OFC (Schoenbaum et al., 1998a; Stalnaker et al., 2014). Therefore, while the OFC may contribute to both types of conditioned response, OFC neurons explicitly encode in their firing patterns the goal-tracking conditioned response that more closely represents the expected outcome.

In addition to measuring OFC firing during conditioned responses, we also measured increases in firing rate immediately following presentation of the reward predictive cue. Multiple studies report OFC excitations to reward predictive cues in both primate and rodent models

(Moorman and Aston-Jones, 2014; O'Doherty et al., 2002; Schoenbaum and Roesch, 2005; Tremblay and Schultz, 1999b), and OFC cells in the present study displayed excitations to both cue presentation and cue offset. Both aspects of the cue provide valuable information about the timing of reward receipt, with cue onset signaling pending reward delivery, and cue offset being the most proximal signal of immediate reward availability. However, it is possible that OFC cells are firing due to salient changes in the testing chamber, and not specifically to stimuli that predict reward. To address this, we analyzed firing in response to house light illumination, which occurs at the beginning of every conditioning session. The house light represents a salient change in the testing environment, but not one that is paired with immediate reward delivery. We found that OFC neurons did not show a time locked excitation to the house light, which stands in contrast to their increased firing rate within the first second after cue presentation. A limitation of the present study is the lack of an unpaired stimulus for comparison, but a recent study that included an unpaired stimulus (Moorman and Aston-Jones, 2014) reported that OFC neurons exhibit reduced excitation to unpaired stimuli relative to reward-predictive stimuli. The inclusion of an unpaired stimulus would also allow us to discern the effects of nicotine on neuronal firing and behavior, as nicotine could enhance the reinforcing properties of an unpaired stimulus. However, we have previously demonstrated that nicotine exposure should not enhance the reinforcing properties of a non-reinforcing stimulus (Palmatier et al., 2012, 2007).

In this study, we found that peak firing rates in NIC animals were lower than those in SAL animals at cue onset and during a goal-tracking conditioned response. When nicotine was not injected prior to session initiation, NIC animals exhibited a higher peak firing to both cue presentation and cue offset. Similar to a previous report (Guy and Fletcher, 2014a), a reduction in conditioned responding accompanied these physiological effects, suggesting that the behavioral and physiological effects of nicotine resulted from acute actions of the drug.

At the time of testing, rats in this experiment had been receiving single daily injections of nicotine, 5 days on and 2 days off, for at least 8 weeks. Animals received enough nicotine to

produce locomotor sensitization (Benwell and Balfour, 1992; Cadoni and Di Chiara, 2000; Miller et al., 2001) but would not have achieved the long-lasting increase in blood concentration of nicotine seen with self-administration. Studies of the effects of chronic exposure to nicotine, either through self-administration of the drug or through passive exposure paradigms, have demonstrated changes on a cellular and behavioral level in humans and animals (Barik and Wonnacott, 2009a; Perry et al., 1999). Acute or low dose administration of the drug can also result in changes to gene expression (Mychasiuk et al., 2013) as well as receptor expression and behavior (Barik and Wonnacott, 2009b; Vezina et al., 2007). Although we cannot be sure of the extent of neuroadaptations induced by nicotine exposure in our paradigm, it is clear that this exposure resulted in physiological adaptations that were alleviated in the absence of nicotine.

Nicotine acts on nicotinic acetylcholine receptors (nAchRs) that are comprised of combinations of receptor subunits that display variations in receptor level physiology resulting in differences in affinity and rates of receptor desensitization or upregulation (Feduccia et al., 2012; Picciotto et al., 2008). NAChRs are located on multiple cell types in the PFC, including fast spiking and non-fast spiking interneurons (Poorthuis et al., 2013). Activation of nAchRs on interneurons can lead to the increase in GABAergic transmission within the PFC (Couey et al., 2007b) and could reduce firing of pyramidal neurons, which would provide an explanation for the reduction in peak firing rate that we observed. We found that inactivation of the OFC by microinfusion of GABAergic agonists resulted in a slight reduction in goal tracking, specifically in NIC animals. This might have resulted from the compounded interaction of nicotine on GABAergic signaling, along with the increase in GABA receptor activation caused by drug infusion. Future studies could utilize techniques that do not target neurotransmitter signaling, such as chemogenetic inactivation of the region immediately before conditioned responding. Overall, we expect that the intricate pattern of nicotinic receptor activation, desensitization, and upregulation led to the nicotine-induced changes in cell firing that we observed, but further

studies are necessary to complete our understanding of the effect of receptor-level plasticity on real-time neuronal firing patterns.

The complex activation profile of nAChRs within the PFC alone could provide an explanation for the physiological results we observed. However, nicotine is capable of acting on nAChRs present throughout the brain, particularly within corticolimbic regions involved in reward processing and motivated behavior (Markou, 2008). Activation of nAChRs within regions that project to the OFC, such as the ventral tegmental area, can influence both behavior and cell firing within the OFC. In the ventral tegmental area, activation of nAChRs on dopaminergic projection neurons can lead to the release of dopamine in the PFC (Livingstone et al., 2009b). With this study, we begin to elucidate the effects of nicotine on phasic firing patterns in the OFC, but additional studies will be required to form a complete picture of the circuit-level effects of nicotine on both behavior and the underlying neuronal activity.

This is the first set of experiments to systematically explore the neurophysiology of nicotine-enhanced sign and goal tracking; therefore, several questions about the effects of nicotine and the neurobiological underpinnings of its effects on motivated behavior remain. For example, is it problematic that nicotine appears to enhance approach to both the sign and the goal in this paradigm? Most substance dependence models have emphasized sign tracking and its role in vulnerability, yet we (Palmatier et al. 2013) have shown that nicotine can increase both sign tracking and goal tracking to a sucrose-paired stimulus, and others (Yager and Robinson, 2015) have shown that sign-and goal-trackers approach a nicotine-paired stimulus equally. In addition, while we have begun to explore the role of the OFC in these behaviors, the recruitment of the broader mesocorticolimbic circuit should also be investigated. Further clarification could be garnered by specifically targeting corticolimbic pathways thought to be associated with this behavior. Lastly, the precise actions of nicotine that lead to the measured behavioral and physiological changes are beyond the scope of this study. Future investigations

of the cellular events, triggered by repeated exposure to nicotine, will bolster our understanding of the process by which nicotine modifies cue-evoked behavior.

Conclusions

Although we utilized an animal model of conditioned responding to probe the ability of nicotine to influence approach to a reward-predictive cue, these results have translational relevance as the same phenomenon has been observed in studies of similar cue-evoked behavior in humans. Specifically, nicotine use can enhance attentional bias to drug-associated cues in smokers (Chanon et al., 2010; Field et al., 2004) and increased cue-related activity in mesotelencephalic systems can predict relapse to nicotine use (Janes et al., 2010; McClernon et al., 2007; Versace et al., 2014). Moreover, smoking nicotine-containing cigarettes can also enhance ratings of facial attractiveness in smokers, relative to smoking de-nicotinized cigarettes (Attwood et al., 2009). In studies of smokers who were presented with both food and cigarette cues and then asked about craving, a strong cue-induced craving for food was correlated with craving cigarettes (Mahler and de Wit, 2010; Styn et al., 2013). Thus, using animal models to investigate sources of biological variability in nicotine's enhancement of incentive stimuli can contribute to potential targets for intervention in the treatment of nicotine addiction.

In summary, using an established model of Pavlovian conditioned approach behavior that is enhanced by nicotine, we have shown that the OFC is recruited primarily during the goal-tracking conditioned response and that nicotine exposure acutely blunts firing in the OFC. These findings further our understanding of the ability of drugs of abuse to amplify existing variation in behavioral and physiological responses to conditioned cues.

Figures

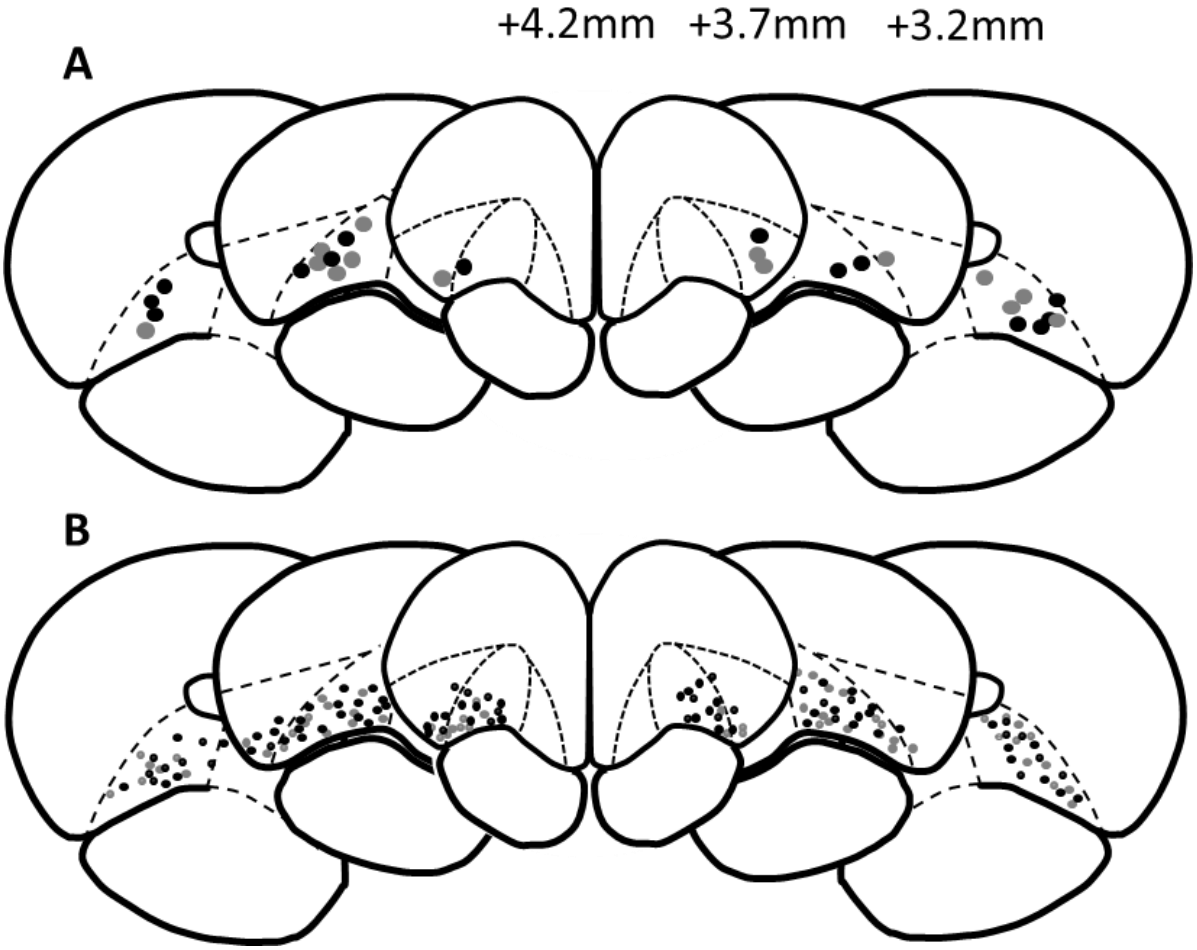

Figure 2.1: Representative cannula and electrode placements in Experiments 1 and 2
Representative schematics of cannula (A) and individual electrode wire (B) placements in rats from Experiments 1 and 2, respectively. AP distances from bregma are indicated in mm. Grey circles represent placements from SAL animals, black circles represent placements from NIC animals. Atlas images are adapted from Paxinos and Watson (1998).

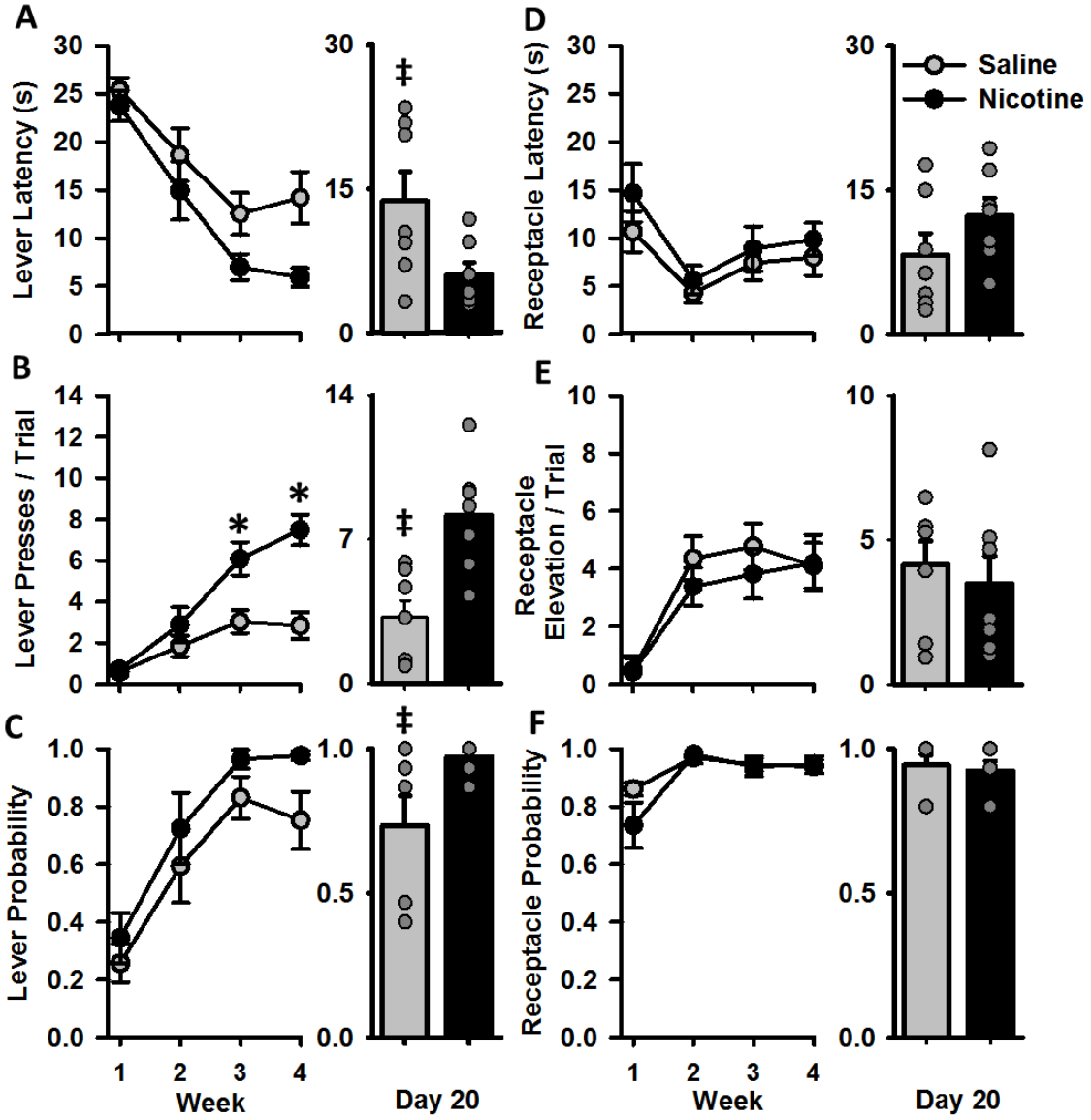

Figure 2.2: Cue evoked behavior during acquisition of Pavlovian conditioned approach behavior for animals in Experiment 1.
Rats were trained for 20 days, data are collapsed across 4 weeks and presented as the mean ± SEM for SAL (grey circles) and NIC (black circles) rats. Figures A-F represent separate measures of sign and goal tracking behavior with bar graphs comparing groups only on the last day of training (Day 20): (A) Latencies to approach the lever (B) lever presses per trial (C) probability of pressing the lever, (D) latency to approach the receptacle (E) receptacle elevation scores per trial (F) probability of entering the receptacle. The right side of each panel depicts group behavior (mean ± SEM) on the last day of training (Day 20). Behavior of individual animals in each group on that day are represented by grey circles (note that some circles overlap, especially in the probability graphs). * week × group interaction $p<0.05$, ‡ difference between NIC and SAL groups on the last day of training, $p<0.05$.

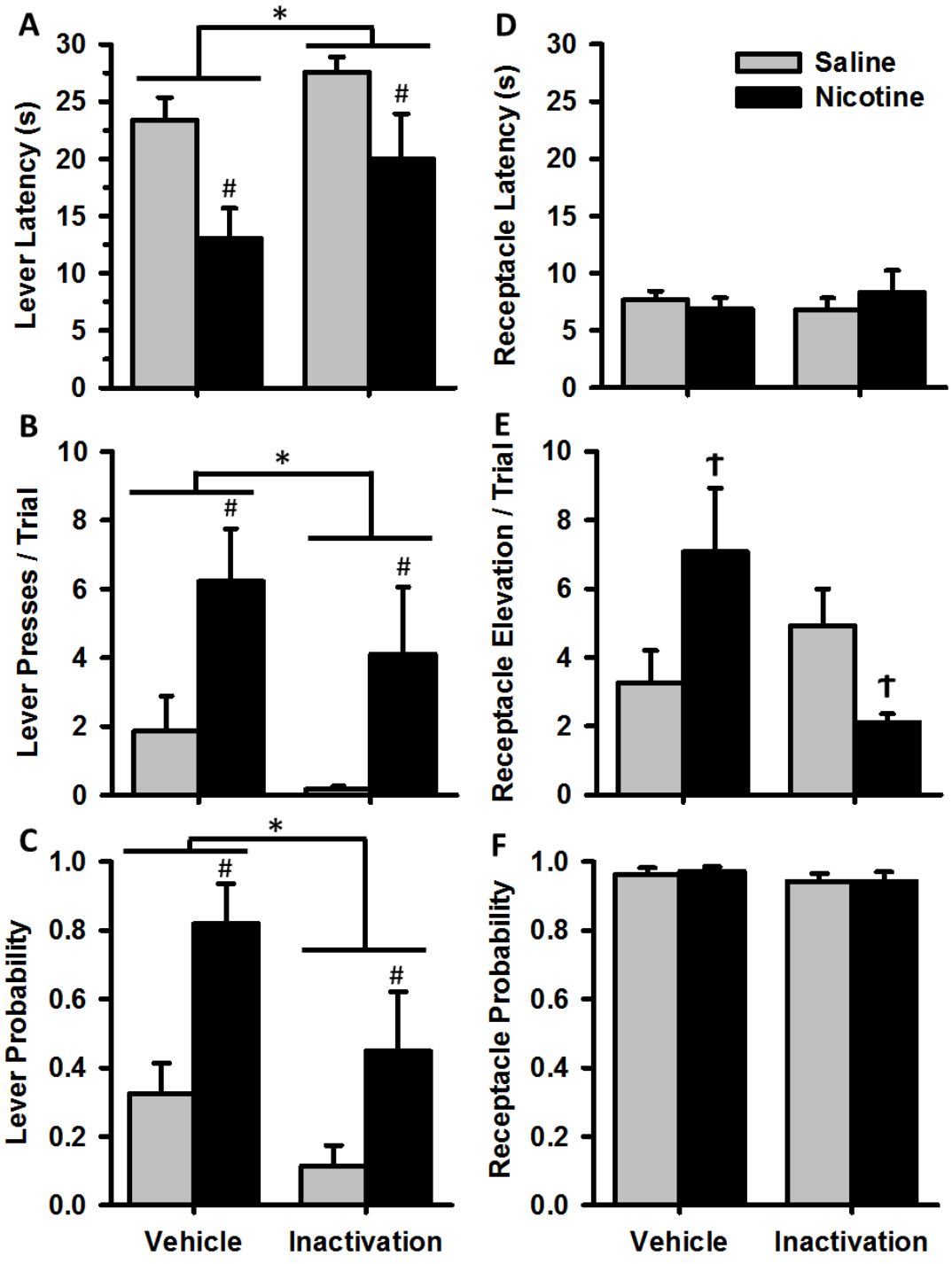

Figure 2.3: Cue evoked behavior after pharmacological inactivation of the OFC.
Data are presented as mean ± SEM for NIC (black bars) and SAL (grey) groups after infusion of either vehicle or the GABA receptor agonists baclofen and muscimol into the OFC. Behavioral measures (A-F) are as described in Figure 2. * main effect of infusion $p<0.05$, # main effect of group $p<0.05$, † group × infusion interaction $p<0.05$.

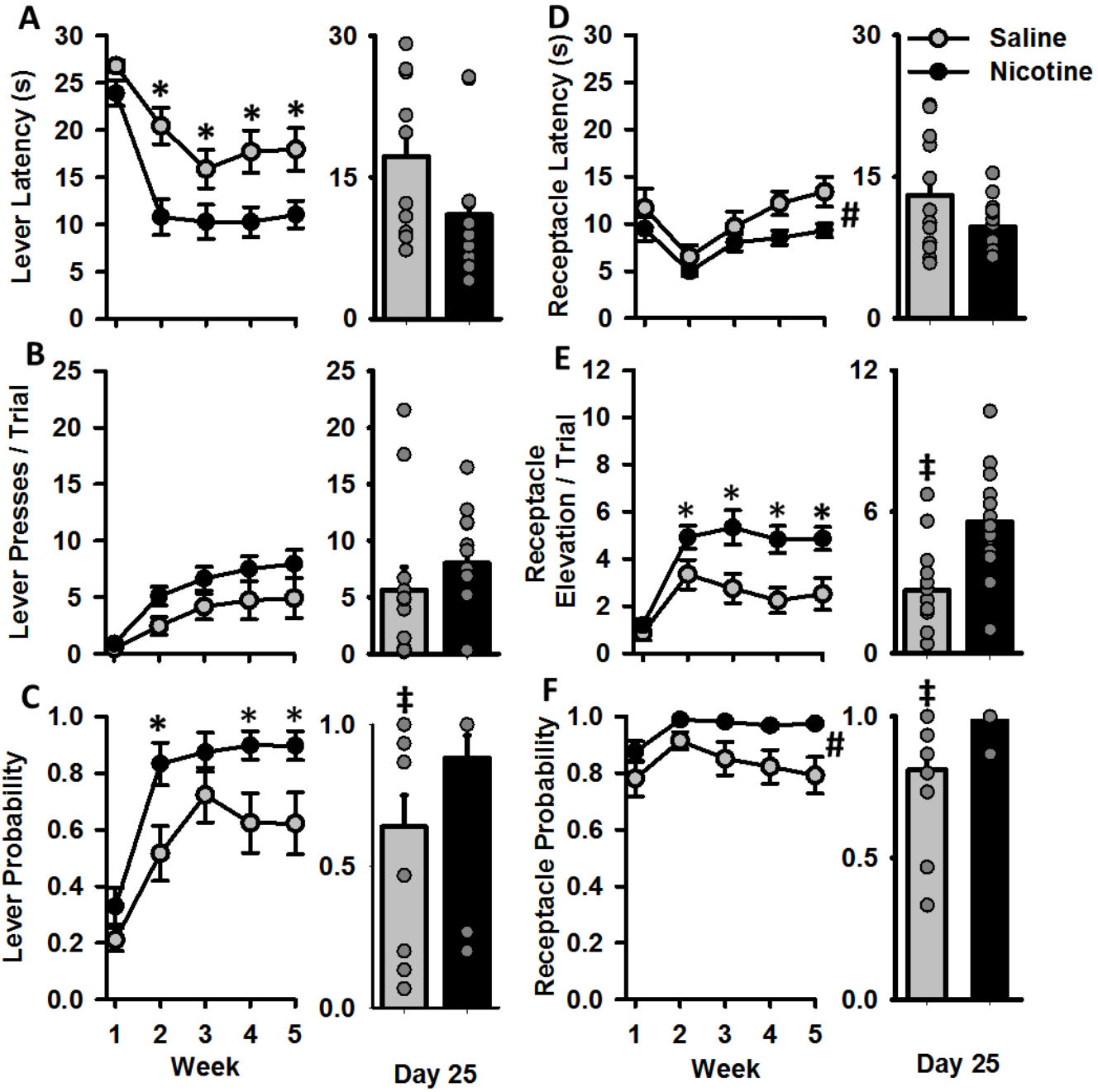

Figure 2.4: Cue evoked behavior during Pavlovian conditioned approach training for animals in Experiment 2.
Rats were trained for 25 days and data are collapsed across 5 weeks and presented as mean ± SEM. Panels A-F depict behavior of NIC (black) and SAL (grey) groups, as described in Figure 2. The right side of each panel depicts group behavior (mean ± SEM) on the last day of training (Day 25). Behavior of individual animals in each group on that day are represented by grey circles (note that some circles overlap, especially in the probability graphs). * Group × week interaction $p<0.05$, # main effect of group $p<0.05$, ‡ difference between groups on Day 25 of training.

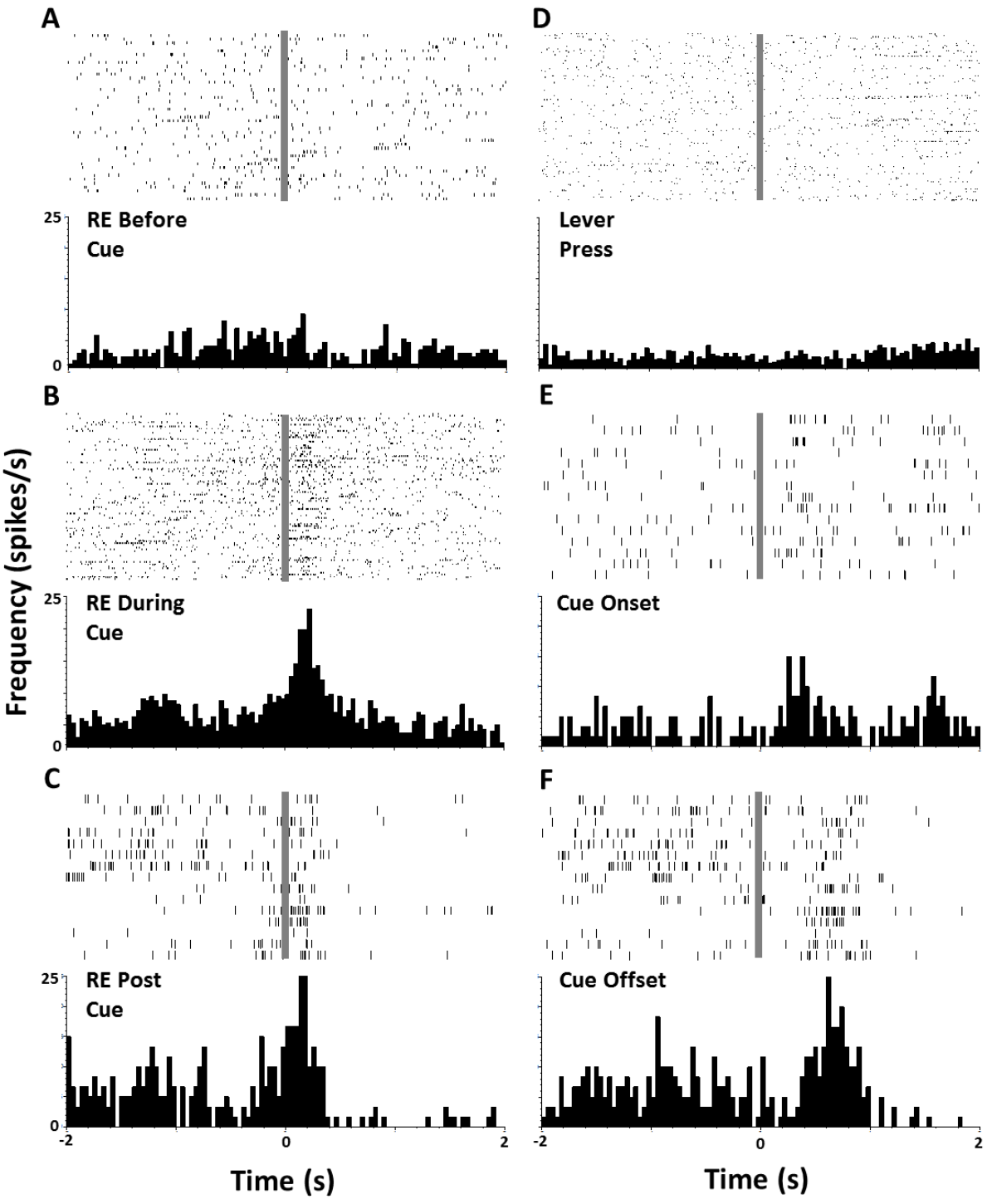

Figure 2.5: Rasters and peri-event histograms depicting phasic activity of one individual example neuron.
Panels A-F represent individual spikes and averaged firing rate during a 4 second period surrounding an event of interest at time=0 s (grey bar). Events of interest are noted on each panel, RE = receptacle entry.

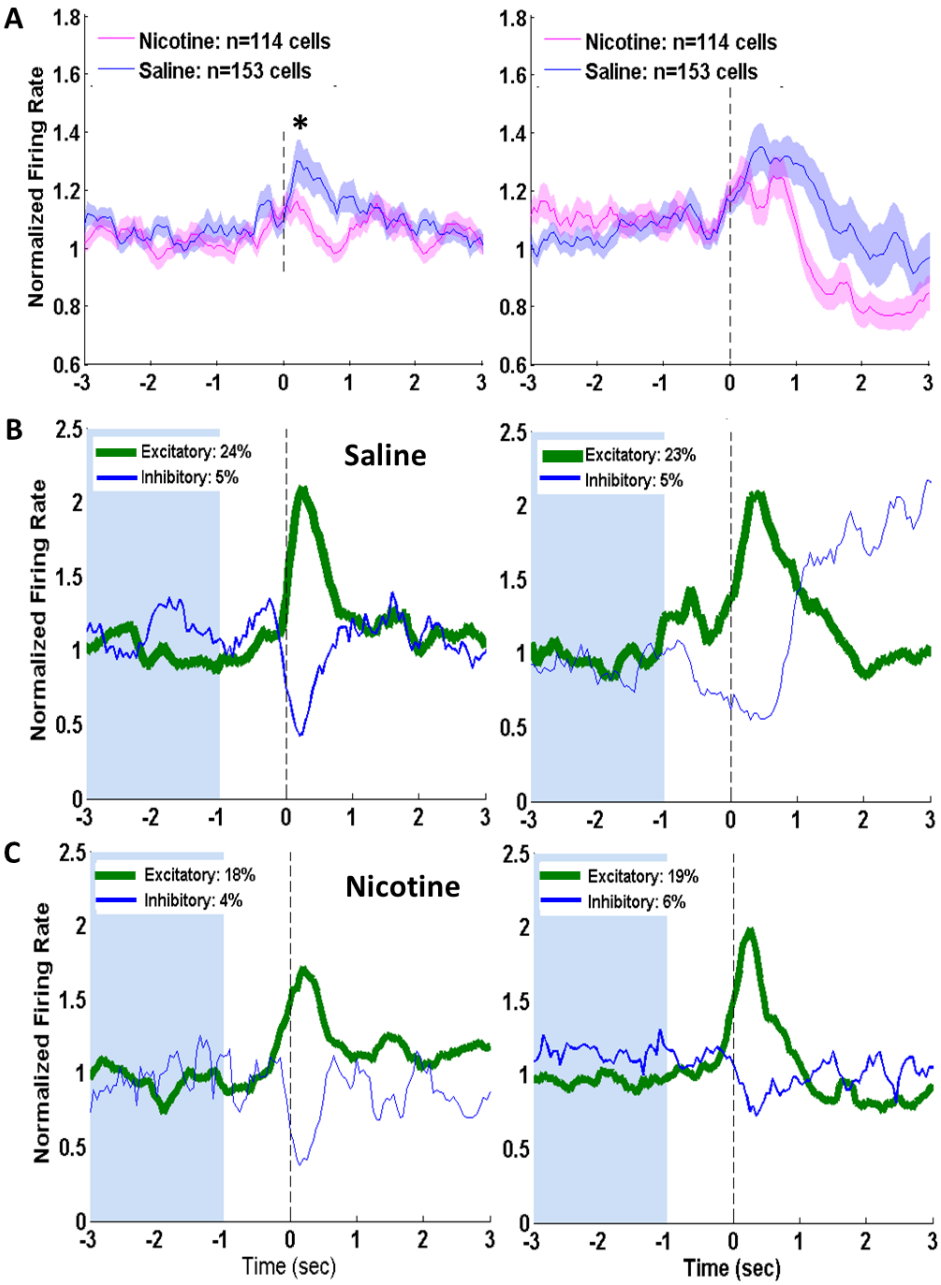

Figure 2.6: Single unit electrophysiology recordings at cue onset or offset during a Pavlovian conditioning session.

(A) Neuronal population activity in the OFC is presented as mean firing rate (±SEM, shaded) and normalized to whole session firing rate for nicotine-exposed (pink) and saline (blue) animals at cue onset and cue offset. (B, C) Phasic firing patterns of neurons that significantly changed their firing rate surrounding either cue offset or cue onset, for SAL (B) and NIC (C) groups. Green histograms represent cells that increased their firing rate, and blue histograms represent cells that decreased their firing rate, line thickness represents the proportion of cells displaying each phasic pattern. (*) significant difference in peak firing rate between groups, $p<0.05$.

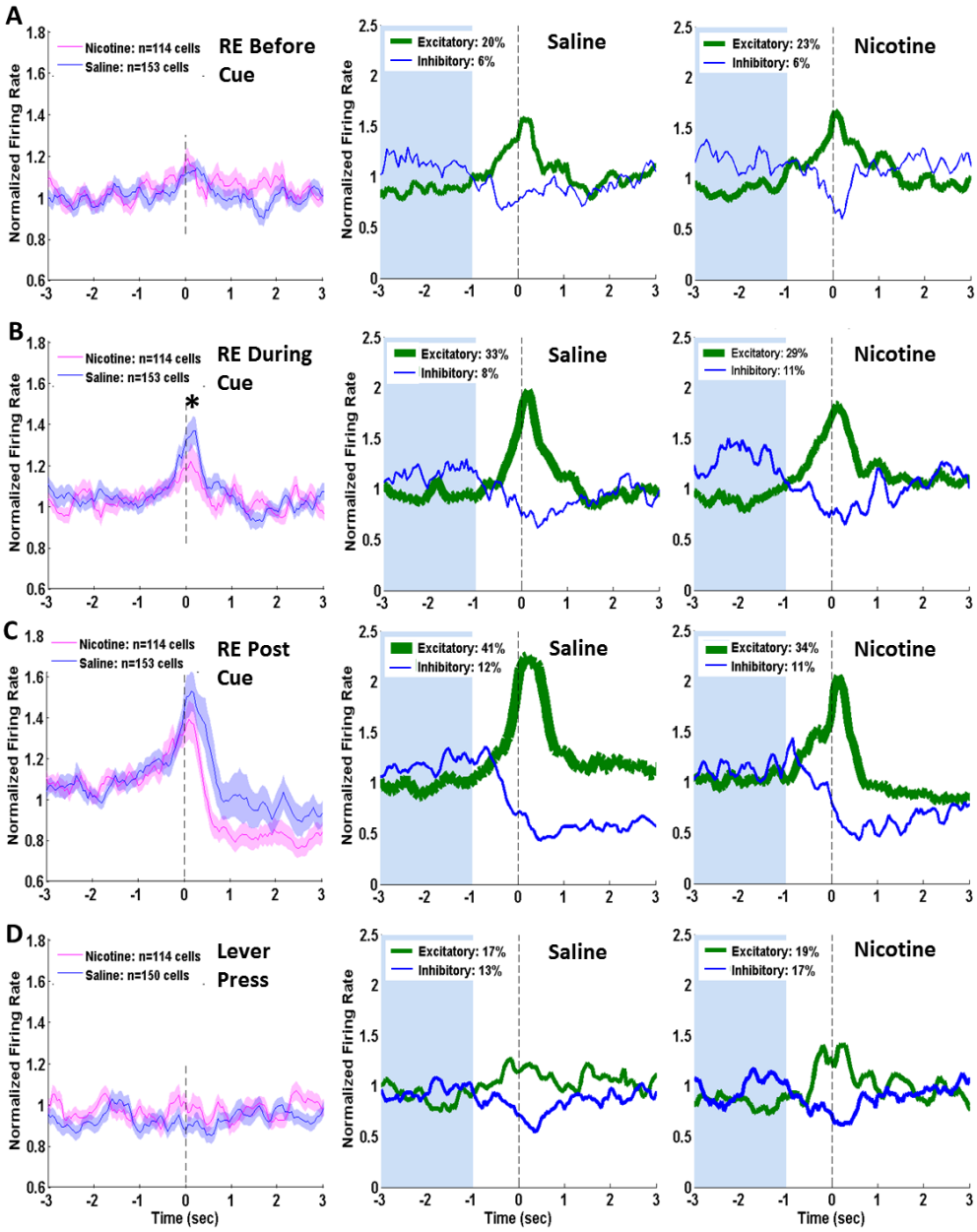

Figure 2.7: Single unit electrophysiology recordings during behavioral responses in a Pavlovian conditioning session.

(A-D, left column) Neuronal population activity in the OFC is presented as mean firing rate (±SEM, shaded) and normalized to whole session firing rate for nicotine-exposed (pink) and saline (blue) animals centered on behavioral responses. Phasic firing patterns of neurons that significantly changed their firing rate surrounding behavioral events are depicted for SAL (center column) and NIC (right column) groups. Green histograms represent cells that increased their firing rate, and blue histograms represent cells that decreased their firing rate, line thickness represents the proportion of cells displaying each phasic pattern. (*) significant difference in peak firing rate between groups, $p<0.05$.

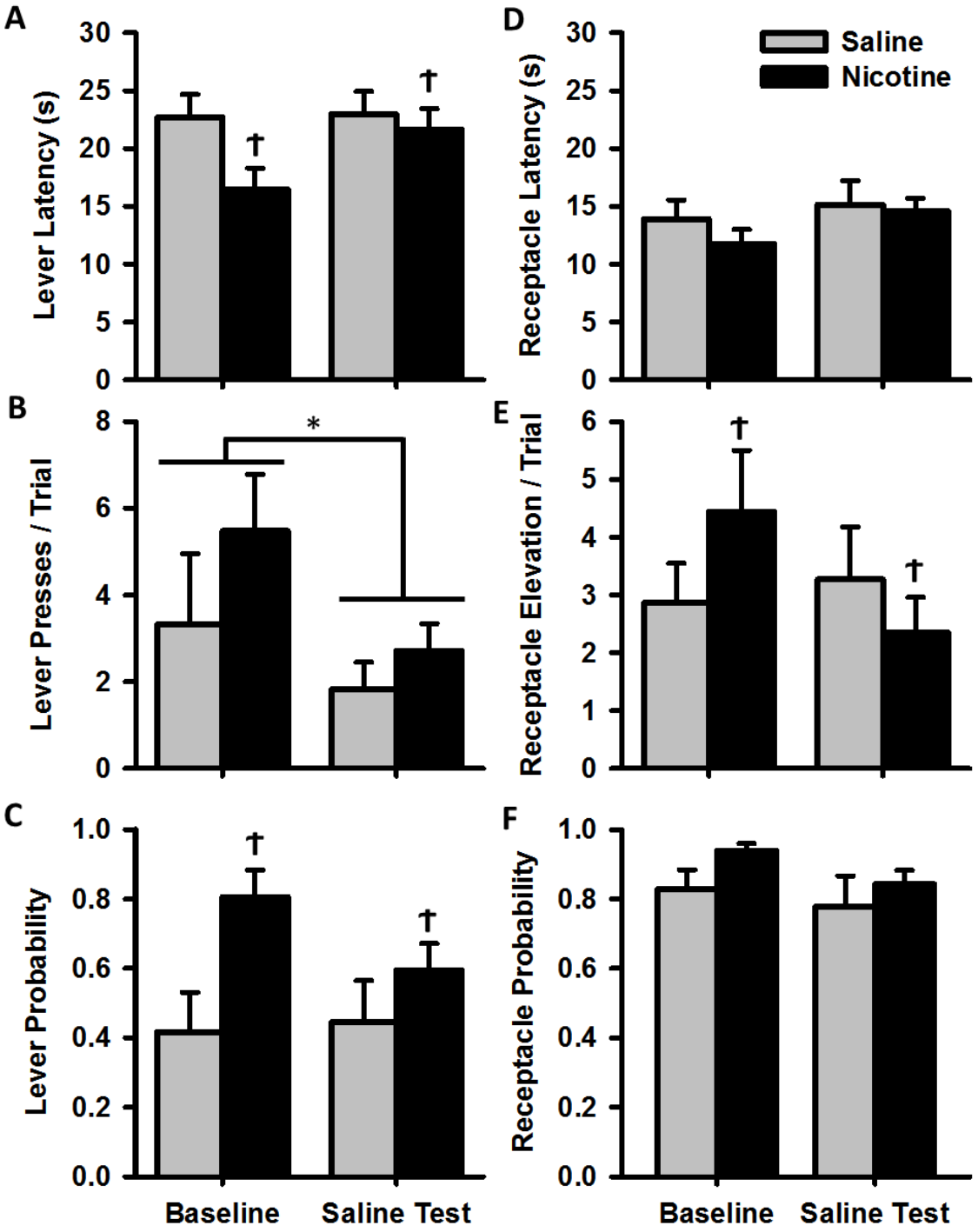

Figure 2.8: Cue evoked behavior during the saline test session.
Data are presented as mean ± SEM for NIC and SAL groups after injection with saline during the saline test session, or on a baseline day in which animals received the assigned drug or control injection. Behavioral measures (A-F) are as described in Figure 2. * main effect of test day $p<0.05$, † group × test day interaction $p<0.05$.

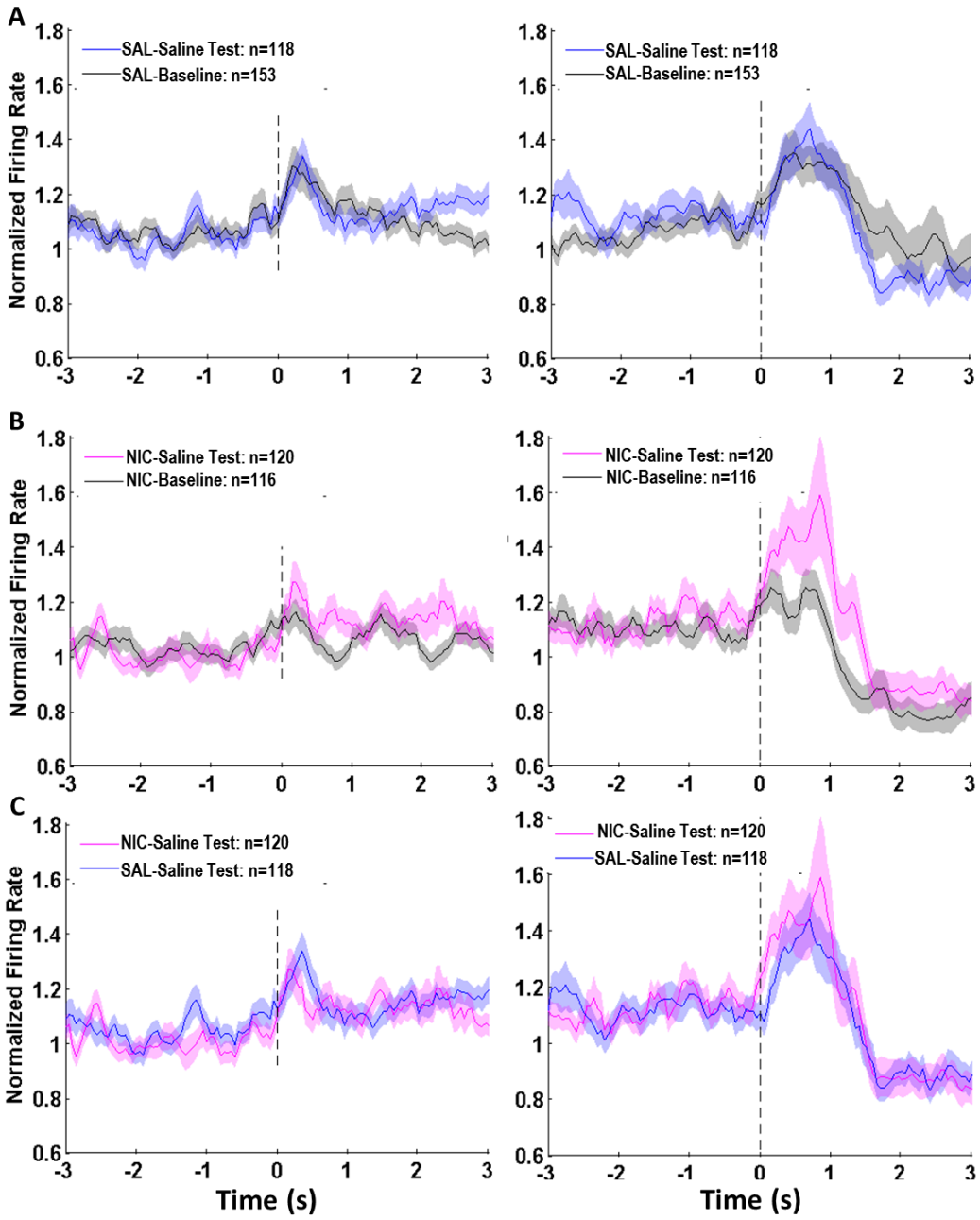

Figure 2.9: Single unit electrophysiology recordings at cue onset or offset during the saline test session.

Neuronal population activity in the OFC is presented as mean firing rate (±SEM, shaded) and normalized to whole session firing rate for nicotine-exposed and saline control animals at cue onset and cue offset. (A, B) Population firing rates in SAL animals on the saline test day (Panel A, blue histograms) and NIC animals (Panel B, pink histograms) compared to the baseline recording session depicted in Figure 6 (grey histograms). (C) Comparison of population activity from SAL (blue) and NIC (pink) animals on the saline test day. (*) significant difference in peak firing rate between groups, $p<0.05$.

Supplemental Figure

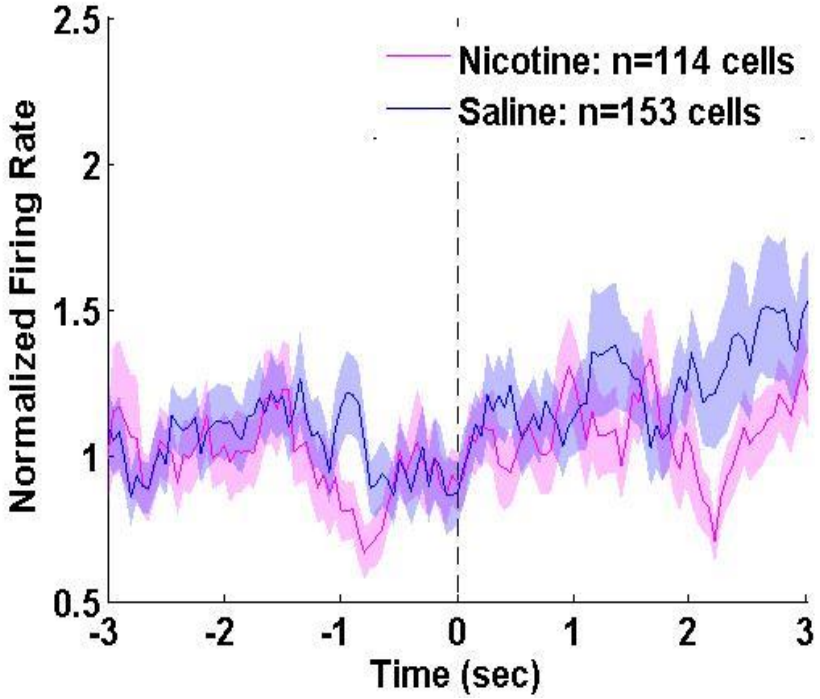

Figure 2.S1: Single unit electrophysiology recordings from OFC neurons upon house light illumination at the beginning of a Pavlovian conditioning session.
House light illumination was a cue that represented general reward availability. Neuronal population activity in the OFC is presented as mean firing rate (±SEM, shaded) and normalized to whole session firing rate for neurons in nicotine-exposed (pink) and saline-exposed (blue) rats. As house light illumination occurred only once during the session, normalized firing rates are not signal averaged across multiple trials. OFC neurons in NIC and SAL groups did not increaser firing rate during the 1 s after house light illumination.

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Chapter 3: Nicotine-enhanced Pavlovian conditioned approach is resistant to manipulation of expected outcome

Introduction

The influence of drug-associated cues is of particular importance to understanding addiction, as exposure to these cues can stimulate craving and precipitate relapse (Childress et al., 1993). Cues can develop strong associations with a drug through Pavlovian learning, during which a conditioned stimulus (CS) is repeatedly paired with an unconditioned stimulus (US), which can be the effects of a drug or a natural reward. Over time this CS can acquire conditioned motivational properties, becoming attractive and able to arouse behavior (Robinson and Berridge, 1993). Multiple drugs of abuse, including nicotine, have been shown to enhance the attribution of incentive salience to a conditioned cue (Palmatier et al., 2013b; Saunders and Robinson, 2011; Tomie et al., 2008). This effect has been measured in smokers using attentional bias tasks, in which smokers allocate excessive attention to stimuli associated with cigarettes or smoking, and this enhanced attentional bias is often correlated with subjective craving (Bradley et al., 2004; Chanon et al., 2010; Field et al., 2009; Mogg et al., 2003).

Pavlovian conditioned approach is a model that can be used to measure the incentive properties of a CS in animals. In this model, animals learn the stimulus-outcome association between the presentation of the CS and the delivery of the US. They begin to exhibit conditioned responses to CS presentation by approaching and interacting with the CS (sign tracking) or the location of US delivery (goal tracking). While the expression of a conditioned response indicates the formation of a predictive relationship between the CS and US, sign tracking is specifically thought to indicate that the CS has become an incentive stimulus (Flagel et al., 2011). Importantly, the enhanced attribution of salience to a CS will emerge in the

absence of drug exposure (Flagel et al., 2007), when drugs are the US (Krank et al., 2008; Peters and De Vries, 2014; Uslaner et al., 2006), or when the animal has been exposed to the drug outside of training (Olausson et al., 2004; Yager and Robinson, 2015). Drugs of abuse promote the attribution of incentive properties to conditioned stimuli, and the expression of this conditioned response is linked to other behaviors that predict addiction vulnerability such as impulsivity, locomotor sensitization, and enhanced drug self-administration (Robinson et al., 2014; Tomie et al., 2008).

Studies of behavioral responses in this type of paradigm primarily focus on animals pre-classified as sign trackers or goal trackers. Sign-tracking animals are thought to show less behavioral flexibility in response to changes in the previously learned stimulus-outcome contingency. While sign trackers perform similarly to goal trackers during extinction of instrumental drug self-administration (Saunders and Robinson, 2011; Versaggi et al., 2016), they are slower to update their behavior during extinction conditions in a Pavlovian task (Ahrens et al., 2016). In one study in which individual animals were trained to exhibit sign tracking and goal tracking to separate stimuli, the sign-tracking behavior was more persistent over multiple extinction sessions and within the first extinction session (Beckmann and Chow, 2015). Thus, it appears that animals classified as sign trackers are less flexible in updating their conditioned behavior when US values change, and sign-tracking behavior may be less flexible than goal tracking regardless of the animals' classification. Exposure to drugs such as nicotine enhances the expression of the sign-tracking conditioned response, but the extent to which drugs further reduce the flexibility of this already inflexible behavior has yet to be established.

Nicotine has relatively weak primary reinforcing characteristics compared to other drugs of abuse, but is chiefly responsible for the rewarding and addictive properties of tobacco (Caggiula et al., 2001). Nicotine self-administration in rodents is achievable with the addition of nonpharmacological stimuli, such as pairing a visual stimulus with nicotine infusion. Nicotine exposure can accentuate the reinforcing properties of non-nicotine stimuli (Olausson et al.,

2004), as well as the incentive properties of cues associated with direct administration of nicotine or a non-nicotine reward. Previous studies have indicated that nicotine possesses incentive-amplifying effects, as exposure promotes the motivational properties of other stimuli (Palmatier et al., 2012). Indeed, nicotine exposure enhances conditioned approach to the US (Olausson et al., 2003; Stringfield et al., 2017) as well as to the CS (Guy and Fletcher, 2014a; Palmatier et al., 2013a; Stringfield et al., 2017; Yager and Robinson, 2015). While it has been established that nicotine promotes conditioned responding and the attribution of salience to a CS, the effects of nicotine on behavioral flexibility are less understood. Nicotine self-administration is capable of transitioning from goal-directed drug-seeking that is sensitive to devaluation, to less flexible, habitual drug-seeking (Clemens et al., 2014). Chronic nicotine exposure also reduces behavioral inhibition in rats trained on a go/no-go task (Kolokotroni et al., 2014). This suggests that nicotine reduces the flexibility of drug-seeking behavioral responses as well as behaviors focused on a natural reward. Less is known, however, about how nicotine influences the flexibility of conditioned responses – and specifically sign tracking – after manipulation of the value of the expected outcome.

The opioid receptor antagonist naltrexone is used in treatment of alcohol use disorders to various efficacy (Garbutt et al., 2014; Maisel et al., 2013). Our lab has previously demonstrated that naltrexone administration is sufficient to reduce goal-directed but not habit-like alcohol seeking, potentially due to the relative flexibility of these two response strategies and the ability of naltrexone to target alcohol reward-focused responding (Hay et al., 2013). Naltrexone has been proposed as a potential treatment method to aid in smoking cessation, as the opioid system is impacted by nicotine (Berrendero et al., 2010) and naltrexone administration may reduce the reinforcing effects of nicotine (Rukstalis et al., 2005). Studies of smokers attempting to reduce cigarette consumption with a naltrexone regimen show variable success (Ahmadi et al., 2003; Covey et al., 1999; O'Malley et al., 2006; Wong et al., 1999). Studies using animal models of naltrexone exposure indicate contrasting effects of the drug on

the reinforcing properties of nicotine. Naltrexone reduces cue-induced reinstatement of nicotine seeking, but not nicotine self-administration (Liu et al., 2009), suggesting that the drug acts on the incentive amplifying properties of nicotine but not its primary reinforcing properties. However, naltrexone only produces slight reductions in nicotine-enhanced conditioned reinforcement, suggesting that the naltrexone does not target the enhanced incentive-salient properties of conditioned cues (Guy and Fletcher, 2014b). Thus, it is not well established if naltrexone acts on the incentive amplifying or reward-associated effects of nicotine, although this information would inform the use of naltrexone for smoking cessation in human populations.

Here, we investigated the extent to which nicotine exposure blunted flexibility in conditioned behavior after reduction in US reward or pharmacological challenge. Specifically, we evaluated behavior after delivery of a new and less valuable reward and under extinction conditions when no reward is delivered. In addition, we tested the effect of naltrexone on conditioned approach in nicotine-exposed and control animals. We hypothesized that animals exposed to nicotine would be slower or less likely to update conditioned responses – particularly the sign-tracking response – after a change in the expected reward or naltrexone challenge.

Methods

Animals

Adult male Sprague Dawley rats (225-250g on arrival) were purchased from Harlan/Envigo (Indianapolis, IN, USA) and pair housed during initial training, then individually housed after surgery. Animals were provided with food and water *ad libitum* during the entire study. Rats were housed in a vivarium on a 12:12 hour light:dark cycle, and experiments occurred during the light cycle. All experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Behavioral training

The behavioral training for both cohorts of animals is described in a previous publication (Stringfield et al., 2017). Animals were assigned to either a nicotine-exposure group (NIC) or a saline-exposure, control group (SAL). Nicotine hydrogen tartrate salt (Sigma-Aldrich, St. Louis, MO) was dissolved in sterile saline and the pH was adjusted to 7.0 ± 0.2. Rats received one injection of 0.4 mg/kg nicotine (s.c., dose calculated from the free base form) or an equivalent volume of saline for two days to habituate them to the injection procedure. The first 4 or 5 weeks of training were conducted in Plexiglas operant chambers (MedAssociates, St Albans, VT), assembled with a recessed reward receptacle, cue light, and retractable lever on one wall of the chamber, and a house light located on the opposite wall. A photobeam detector was positioned across the reward cup to detect head entries into the receptacle. Animals were habituated to the testing chambers during one day of receptacle training, in which they were injected with nicotine or saline, returned to their home cage for 10 min, then placed in the testing chamber for 5 min before session initiation. During this session, 20% sucrose was dispensed into the receptacle on a VI120s schedule and head entries were recorded. Thereafter, 20 or 25 Pavlovian conditioning sessions were conducted, Monday-Friday, in which the animals were injected with nicotine or saline 15 min before session initiation as described above. The house light was illuminated throughout the session, and 15 cue-reward pairings occurred on a VI120s schedule of reinforcement. The cue consisted of illumination of the stimulus light and extension of the lever located directly below the light. Cue presentations lasted 30s, and were immediately followed by 0.1ml of 20% sucrose dispensed into the reward receptacle. Lever deflections and head entries into the reward receptacle were recorded but had no programmed consequences. After training, all animals were habituated to custom built plexiglass chambers inside sound attenuated boxes for an additional 5 days before surgery to implant guide cannula or microelectrode arrays (used for a separate study, Stringfield et al., 2017). Prior to the experiments described below, animals in Experiment 3 received bilateral microinfusions of the $GABA_A$ and $GABA_B$ agonists muscimol

and baclofen, and the results of those studies have previously been reported (Stringfield et al., 2017).

Experiments 1 and 2: Water substitution and extinction challenge sessions

In the first cohort of animals, two challenge sessions were conducted to test the flexibility of conditioned approach in NIC and SAL animals. Animals were tethered and electrophysiology recordings occurred during each test session; those data are not reported here. All animals completed both challenge sessions: an 'Extinction' session in which the sucrose reward was withheld after cue offset, and a 'Water' session in which water was dispensed after cue offset instead of the expected sucrose reward. The order of the challenges were counterbalanced across rats and challenge sessions were separated by at least 3 standard Pavlovian sessions, to confirm that conditioned behavior returned to baseline after testing.

Experiment 3: Effect of naltrexone on nicotine-enhanced conditioned approach

In a second cohort of animals, we determined the dose-response effect of naltrexone (Sigma-Aldrich) on conditioned approach. After the previously described microinfusions, rats in Experiment 3 received 2-3 days of standard Pavlovian conditioning sessions to confirm behavior had returned to baseline before the present experiment began. Animals received an injection of each dose of naltrexone (0.3, 1.0, and 2.0 mg/kg) or saline vehicle in a randomized order, 30 min prior to session initiation. Animals were injected with the assigned saline or nicotine solutions and then underwent Pavlovian conditioning sessions as previously described.

Data analysis and statistics

Behavioral measures were compared across exposure group (NIC, SAL) and within each group. Conditioned approach was measured using the latency to approach both the receptacle and the lever, the total lever presses per trial or receptacle entries per trial, and the probability of entering the receptacle or pressing the lever at least once per trial. In addition, as the receptacle was always present (while the lever was only available as part of the CS), a receptacle elevation score was computed by taking the number of receptacle entries that

occurred during the 30-s cue presentation and subtracting from that the number of receptacle entries that occurred during a 30-s period immediately before cue presentation.

For Experiments 1 and 2, behavioral measures were compared across SAL and NIC groups for each challenge session and its corresponding baseline day by using 2-way repeated-measures ANOVA. Tukey's HSD was used for post-hoc comparisons when applicable. In addition, to compare changes that occurred within a session, behavioral measures within each group were analyzed during the first 3 trials and last 3 trials of each test session by 2-way repeated-measures ANOVA followed by Tukey's HSD post-hoc comparisons.

In Experiment 3, given the low N and high variability within groups, we calculated the linear slope of the dose response curve over each dose of naltrexone and vehicle for each individual animal, and then analyzed the difference of the slope from 0 by one-sample t-tests (Hay et al 2013). With this analysis, a slope of 0 represents the null hypothesis that naltrexone has no effect on conditioned approach at any dose tested.

Results

Experiment 1: Effect of water substitution on nicotine-enhanced conditioned approach

Training behavior for this cohort of animals was published previously (see Fig. 4 in Stringfield et al, 2017), during which nicotine exposure enhanced both sign-and goal-tracking conditioned responses. Here, we conducted a test session in which water was delivered in place of the expected sucrose reward. Both NIC and SAL groups showed slight decreases in conditioned approach. Behavior during the water test and corresponding baseline session was first analyzed over the whole session to compare the overall change in behavior between NIC and SAL exposed animals (Figure 1). For lever latency, there was a main effect of group [$F_{(1,23)}$ =9.4, $p<0.01$] and of session [$F_{(1,23)}$ =6.8, $p<0.05$], as NIC animals showed a shorter latency to press the lever, and both groups of animals slightly increased the latency to press the lever during the water session. Rats in both drug exposure groups reduced their lever pressing, reflected in a main effect of session for lever presses, [$F_{(1,23)}$ =9.4, $p<0.01$], along with a main

effect of drug exposure group for lever probability [$F_{(1,23)}=16.2$, $p<0.01$] as NIC rats were more likely to press the lever. Other main effects in group-by-session interactions did not reach significance. For goal tracking behaviors, main effects of session were present for receptacle latency [$F_{(1,23)}=31.1$, $p<0.01$], receptacle elevation score [$F_{(1,23)}=4.3$, $p<0.05$], and receptacle probability [$F_{(1,23)}=21.4$, $p<0.01$] in which both NIC and SAL rats reduced their goal tracking behaviors during the water substitution session. No group differences or group-by-session interactions emerged for goal tracking behaviors.

To further investigate changes in behavior that occurred during the test session, behavioral responses were compared during trial blocks of the first 3 trials and last 3 trials of the baseline and water substitution test sessions within each drug exposure group (Figure 2). For rats in the NIC group, analysis by trial block yielded a main effect of session for lever presses [$F_{(1,12)}=4.9$, $p<0.05$], driven primarily by an increase in lever pressing throughout the baseline session but not the water substitution session. There was also a main effect of trial block on receptacle latency [$F_{(1,12)}=9.1$, $p<0.05$], as animals increased the latency to enter the receptacle on both baseline and test days, but no other effects emerged. SAL rats exhibited reductions in goal-approach behaviors only, as there were trial block by session interactions for latency to receptacle entry [$F_{(1,11)}=6.2$, $p<0.05$] and elevation score [$F_{(1,11)}=4.9$, $p<0.05$]. Post-hoc comparisons indicated an increase in latency to approach the receptacle between the first and last trial blocks on the water test day, as well as a longer latency to approach during the last trial block of the water test session compared to the baseline session ($p<0.01$). While elevation scores increased across the session at baseline and decreased during the water tests session, no pairwise multiple comparisons reached significance.

Experiment 2: Effect of extinction conditions on nicotine-enhanced conditioned approach

During the extinction test session, animals were presented with the cue but sucrose delivery did not occur. Rats in both the NIC and SAL groups reduced conditioned approach during this test session, with the greatest change in behavior occurring in the SAL group.

Results of the whole-session comparisons of extinction test to the baseline day (Figure 3) indicate that NIC rats were faster to press the lever [main effect of group, $F_{(1,22)} = 5.3$, $p<0.05$] and more likely to press the lever [main effect of lever probability, $F_{(1,22)} = 9.6$, $p<0.01$] but no main effects of extinction day. There was not, however, any significant effect of exposure or the extinction test on the number of lever presses per trial. For goal tracking behaviors, there was an effect of extinction on receptacle elevation score [$F_{(1,22)} = 28.7$, $p<0.001$] in which both groups of animals reduced this behavior, but no difference between exposure groups. Group × session interactions were significant for receptacle latency [$F_{(1,22)} = 7.9$, $p<0.05$] and receptacle probability [$F_{(1,22)} = 7.9$, $p<0.05$]. Post-hoc comparisons indicated that both SAL and NIC animals increased the latency and decreased in probability to enter the receptacle on the extinction day ($p<0.01$), and that while the two groups did not differ on the baseline day, there was difference in latency and probability between SAL and NIC groups on the extinction day with SAL animals showing an increased latency and lower probability ($p<0.001$).

Further within-group analysis of behavior across trial block yielded additional effects during the extinction test session (Figure 4). NIC animals showed reduced goal tracking but not sign tracking behaviors, evident in a trial block × session interaction for receptacle elevation scores [$F_{(1,11)} = 5.9$, $p<0.05$], in which the last 3 trials on the extinction day differed from the same trial block on the baseline day ($p<0.05$) and there was a decrease in elevation score across the beginning and end of the session only on the extinction day ($p<0.01$). In addition, there was main effect of session on receptacle latency in which NIC animals increased the latency to enter the receptacle [$F_{(1,11)} = 11.3$, $p<0.01$]. Sign tracking behavior did not change in NIC animals across trial blocks or sessions.

Animals in the Sal group showed stronger changes in both goal-and sign-tracking behaviors. For goal tracking behaviors, there was a trial block × session interaction for receptacle latency [$F_{(1,11)} = 4.9$, $p<0.05$] due to an increase in receptacle latency within the extinction session ($p<0.05$), between the first trial blocks on the extinction and water day

($p<0.05$) as well as the last trial blocks on these days ($p<0.001$). There was a trial block × session interaction for receptacle elevation score [$F_{(1,11)} = 7.3$, $p<0.05$] due to a reduction in elevation score in the last trial block between the extinction and baseline day ($p<0.001$). Trial block × session interactions also emerged for lever latency [$F_{(1,11)} = 6.0$, $p<0.05$] and lever presses per trial [$F_{(1,11)} = 4.9$, $p<0.05$]. Lever latency increased significantly during the extinction session but not the baseline session ($p<0.001$). Lever presses decreased from the first 3 trials to the last 3 trials of the extinction session ($p<0.01$), and there was a difference in lever presses between the last 3 trials of the baseline day and the extinction day ($p<0.05$).

Experiment 3: Effect of on nicotine-enhanced conditioned approach

Training behavior for this cohort of animals was published previously (see Fig. 2 in Stringfield et al, 2017), during which nicotine exposure enhanced primarily sign-tracking conditioned responses. In this experiment, rats were exposed to naltrexone to evaluate the influence of this drug on Pavlovian conditioned approach. A total of 12 rats (6 NIC and 6 SAL) received 3 doses of naltrexone and vehicle, in a randomized order and within-subject design. Naltrexone reduced receptacle-directed responses, and this effect occurred primarily in the NIC-exposed group. We analyzed the slope of the dose response curve for each individual rat across the doses of naltrexone within groups, and found that only NIC animals exhibited a decrease in behavior with increasing dose of naltrexone (Table 1). NIC animals reduced receptacle entries per trial [$t_5 = -2.6$, $p<0.05$] and increased latency to press the lever [$t_5 = 3.6$, $p<0.05$]. There was a trend toward a decrease in receptacle elevation score, but it did not reach significance [$t_5 = -2.3$, $p=0.07$]. Rats did not exhibit any significant change in slope on sign tracking behaviors, and there was no significant change in slope for SAL animals on any behavioral measure.

Discussion

In this study, we investigated potential modifiers of nicotine-enhanced conditioned responding by behavioral and pharmacological means. We investigated the flexibility of

Pavlovian approach behaviors by manipulating the expected outcome predicted by a conditioned cue using water substitution and extinction training. NIC and SAL animals moderately reduced conditioned responding after water substitution, and greatly reduced conditioned responding during the extinction session. SAL animals reduced both sign-and goal-tracking approach behaviors, while NIC animals only reduced goal tracking. In addition to behavioral manipulations, NIC and SAL animals were exposed to multiple doses of naltrexone and evaluated on behavioral measures of Pavlovian conditioned approach behavior. The highest doses of naltrexone moderately reduced goal tracking behaviors in NIC animals, and did not influence sign tracking in either group. These studies reflect a dissociation in the flexibility of conditioned approach seen in rats that are capable of expressing either response upon CS presentation, particularly that sign tracking is resistant to adaptation after behavioral and pharmacological manipulation.

We first altered the expected outcome during Pavlovian conditioning sessions to disturb the learned predictive relationship between the CS and expected reward. During the water substitution test, we manipulated the expected outcome by delivering water instead of the expected sucrose solution. Substitution with water provides a liquid reward, potentially conserving the previously developed consummatory behavior for licking a solution. Rats are able to discriminate between sweetened solutions that differ by a small percentage of sweetener (Sanjuán et al., 2014), suggesting that this water reward would be perceived as different and possibly less valuable as the animals had water freely available in their home cage. A lack of reduction of approach on most measures of behavior suggests that nicotine-and saline-exposed animals only slightly reduce goal-and sign-tracking conditioned responses when they are still receiving a reward, even though the value of that reward has changed. SAL animals showed no change in lever pressing and minimal decreases in goal tracking. NIC rats showed a difference in lever pressing during the water substitution test compared to baseline, the only time these animals demonstrated a change in lever pressing, but this effect was primarily driven by an

increase in lever pressing throughout the baseline session that did not occur during the water substitution session. Thus, reduction in the value of the reward only resulted in a nominal change in conditioned approach.

In contrast to the water substitution day, withholding of the expected US produced large changes multiple measures of approach behavior. Pavlovian extinction was conducted over one test session, and we were able to detect adaptations in behavior during this single session. While both groups reduced goal tracking behaviors, NIC animals specifically showed no change in sign tracking behaviors, compared to controls. This is consistent with prior reports of extinction of sign-and goal-tracking conducted over multiple days of extinction training that detected differences in sign-and goal-trackers on the first day of extinction and on subsequent test days. In a study in which the rats were trained to discriminate between rewarding and nonrewarding blocks of trials, goal trackers were much more likely to reduce behavior specifically during non-rewarded periods than sign trackers (Ahrens et al., 2016). Beckmann and Chow, (2015) also demonstrated that in rats trained to sign track to a lever stimulus and goal track to a separate tone stimulus, the sign-tracking behavior was resistant to extinction over multiple sessions, and this difference was evident on the first day of extinction training. The present study adds to this differentiation between sign and goal tracking behaviors by concurrently measuring the expression of both behaviors during Pavlovian conditioning sessions in which animals typically express both conditioned responses, but to different degrees. We see that not only are animals who are inherently sign trackers less likely to change their behavior, but the behavior itself is resistant to updating.

The combination of these results and previously published studies suggest a difference in the flexibility of sign- and goal-tracking behaviors. Responding despite reward devaluation is a hallmark characteristic of habitual behaviors, and while sign tracking would not be classified as “habitual”, it does overlap by being inflexible after a change in the value of an expected outcome. Sign tracking is resistant to reward devaluation (Morrison et al., 2015; Nasser et al.,

2015), and can continue even in the absence of the US delivery. Sign tracking is resistant to extinction, potentially because the cue has become an incentive stimulus and is able to act as a conditioned reinforcer (Guy and Fletcher, 2014a; Yager and Robinson, 2015). Devaluing this cue requires a reduction in the attributed incentive value of the cue itself, separate from the reward. Nicotine enhances the attribution of salience to the conditioned cue due to its well documented reinforcement-enhancing and incentive-amplifying effects (Palmatier et al., 2006), making the behavior even less flexible in animals exposed to nicotine.

To continue to manipulate conditioned approach behavior, we utilized a pharmacological method and administered multiple doses of the opioid antagonist naltrexone, as this drug may specifically influence NIC-enhanced conditioned approach. Our results indicate that naltrexone reduces goal tracking but not sign tracking, suggesting that naltrexone is not targeting the nicotine-enhanced incentive properties of a conditioned cue, but is influencing the motivational properties of the expected sucrose reward. The mechanism of action of naltrexone for its effects on smoking cessation and nicotine seeking have yet to be concretely identified. In rats, naltrexone does not reduce nicotine self-administration (Corrigall and Coen, 1991; Liu et al., 2009) but does reduce cue induced reinstatement of nicotine seeking (Liu et al., 2009).This suggests that naltrexone may primarily influence the increased incentive and reinforcing properties attributed to nicotine-associated cues. However, in animals exposed to nicotine that have attributed salience to a water-predictive CS, naltrexone does not reduce conditioned reinforcement of the conditioned cue (Guy et al., 2014). Thus, the influence of naltrexone on the enhanced cued properties of nicotine requires further study.

Naltrexone typically reduces seeking of other drugs at a dose that does not influence sucrose seeking (Burattini et al., 2008; Häggkvist et al., 2009; Hay et al., 2013). While it is possible that the high dose of naltrexone directly reduced the rewarding value of sucrose, that does not appear to be the case as the control animals were not affected. Naltrexone specifically blocked the conditioned approach-enhancing effects of nicotine, and selectively targeted goal-

approach without affecting approach to the CS. This suggests a differentiation between sign- and goal-tracking conditioned responses, particularly in the role of the opioid system, that warrants further study.

A limitation of the naltrexone experiment is small sample size, particularly for measuring conditioned responses on the Pavlovian approach paradigm. Many studies that separate animals into sign-and goal-trackers use much larger groups, particularly because of the increased number of animals needed to accurately measure the expected individual variability and group them based on behavioral phenotype (Meyer et al., 2012).Thus, any follow-up study would benefit from the increased power to consider individual variation at and identify more significant differences in behavioral response to naltrexone.

Ongoing evaluation of the formation and flexibility of stimulus-outcome associations is important for potential treatments of nicotine abuse, as nicotine replacement therapy alone may not target the enhanced salience of drug-associated cues that can lead to craving and relapse (Tiffany et al., 2000; Waters et al., 2003). Combined treatment strategies that weaken the nicotine-enhanced associations between environmental stimuli and the drug may strengthen cessation attempts (Caggiula et al., 2001; Rose, 2006; Rose and Levin, 1991). Moving forward, evaluating the neurocircuitry involved in mediating these associations, and the conditioned behavioral responses that develop as a result, is of particular interest for clinical and preclinical research. This study investigated the effect of an opioid antagonist on nicotine-enhanced conditioned responding, and assessing the involvement of opioid receptors both in mediating the incentive amplifying effects of nicotine and in the expression of conditioned responses is of importance in studies of drug addiction and behavior (Berrendero et al., 2010; DiFeliceantonio and Berridge, 2016; Peters and De Vries, 2014; Yager and Robinson, 2015). In clinical populations, evaluation of the *OPRM1* gene polymorphism conveying reduced μ opioid receptor protein is linked to smoking outcomes, the perception of nicotine's subjective rewarding properties, and addiction liability (Ray et al., 2006; Schwantes-An et al., 2016). Continuing to

elucidate the effects of opioids on nicotine behavior will be of sustained importance for understanding the effect of nicotine on reward on a neurobiological level.

In this study, we demonstrate that manipulation of the expected outcome reduced conditioned responding in control animals, but nicotine-enhanced responses were less flexible. We also saw that naltrexone can target the goal tracking conditioned responses that are specifically enhanced by nicotine, but does not influence the increased salience of the conditioned cue. Thus, future treatment strategies to decrease nicotine abuse will require the development of strong means to disrupt these stimulus-outcome associations, to reduce the ability of cues to elicit craving and lead to relapse.

Figures

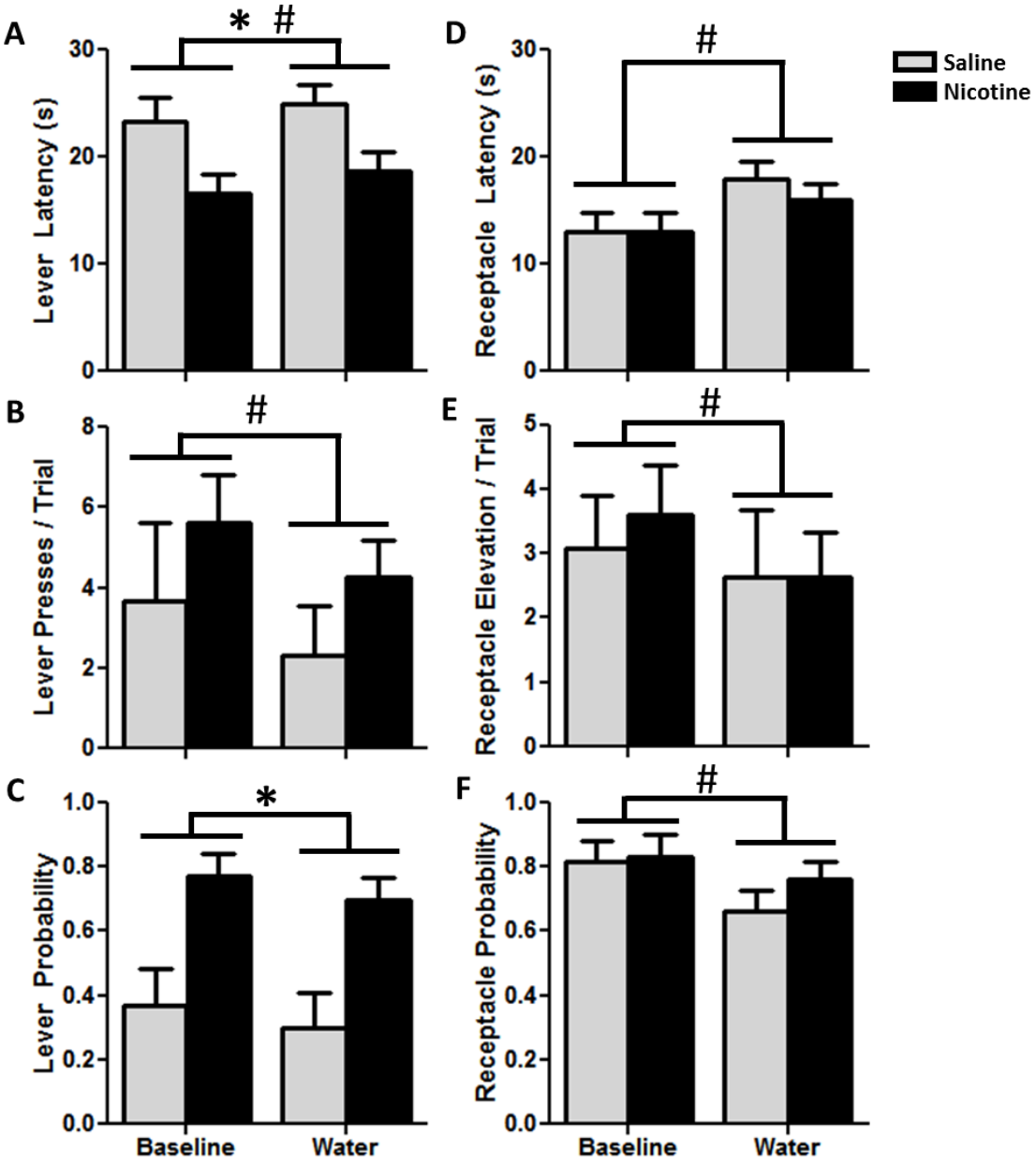

Figure 3.1: Substitution of water for sucrose US reduced conditioned approach over the whole session.

Behavioral measures were compared on a baseline day and the water substitution day in NIC and SAL groups of rats. Measures of sign tracking and goal tracking (mean ± SEM) are displayed as latency to press the lever (A) or enter the receptacle (D), lever presses per trial (B) or receptacle elevation score per trial (E), probability of pressing the lever (C) or entering the receptacle (F). * Main effect of drug exposure, # main effect of water substitution day, $p<0.05$ for all analyses.

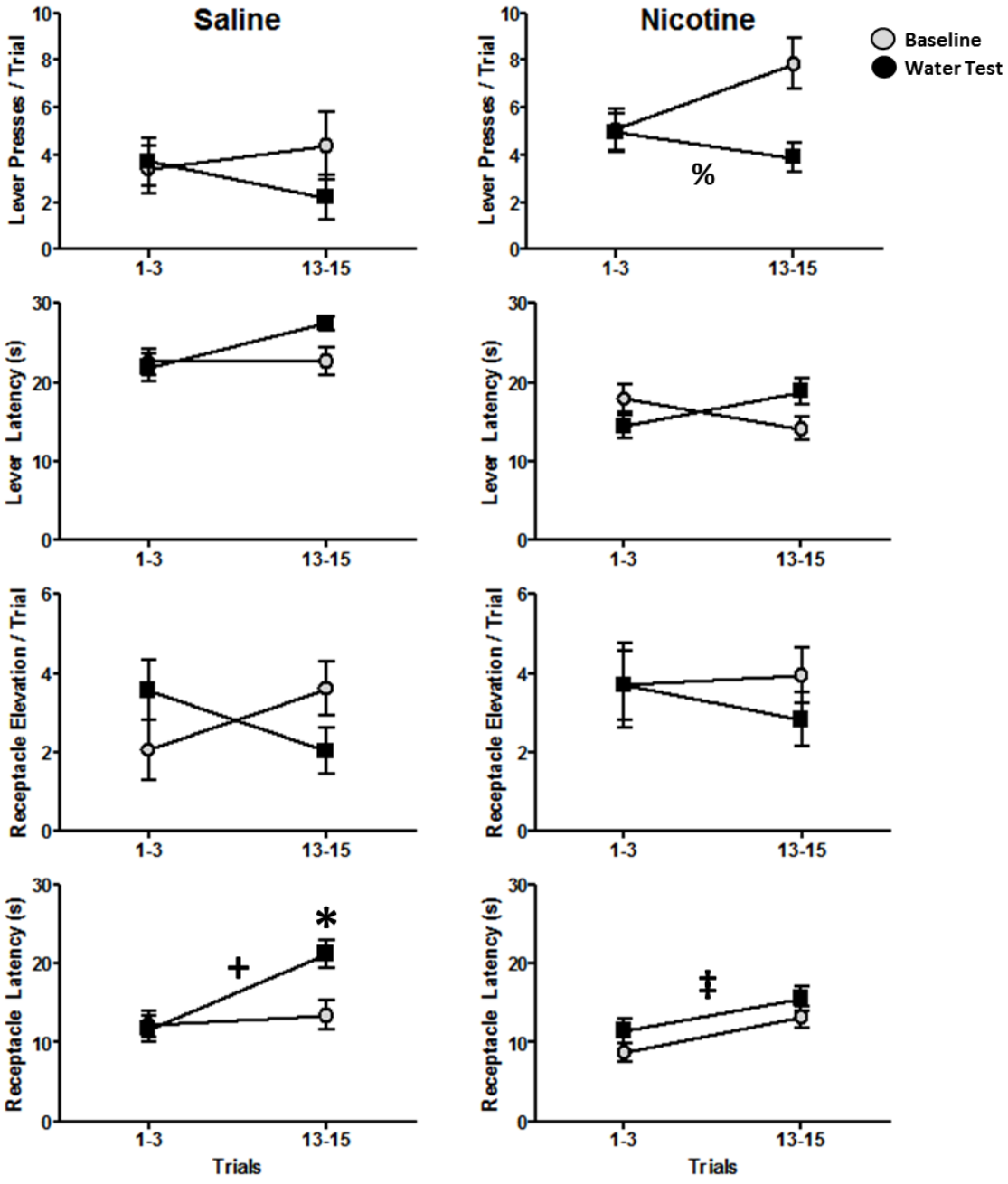

Figure 3.2: Substitution of water for the expected sucrose reward minimally influenced sign-and goal-tracking behaviors within the session.
Conditioned responding during the baseline and water test sessions was compared within drug exposure group at the beginning of the session (Trials 1-3) and end of the session (Trials 13-15). Behavioral measures are as described in Fig 1. % main effect of water substitution day, ‡ main effect of trial block, + interaction, difference between first and last trials on water substitution day, * interaction, difference between Trials 13-15 on the baseline and test days, $p<0.05$ for all analyses

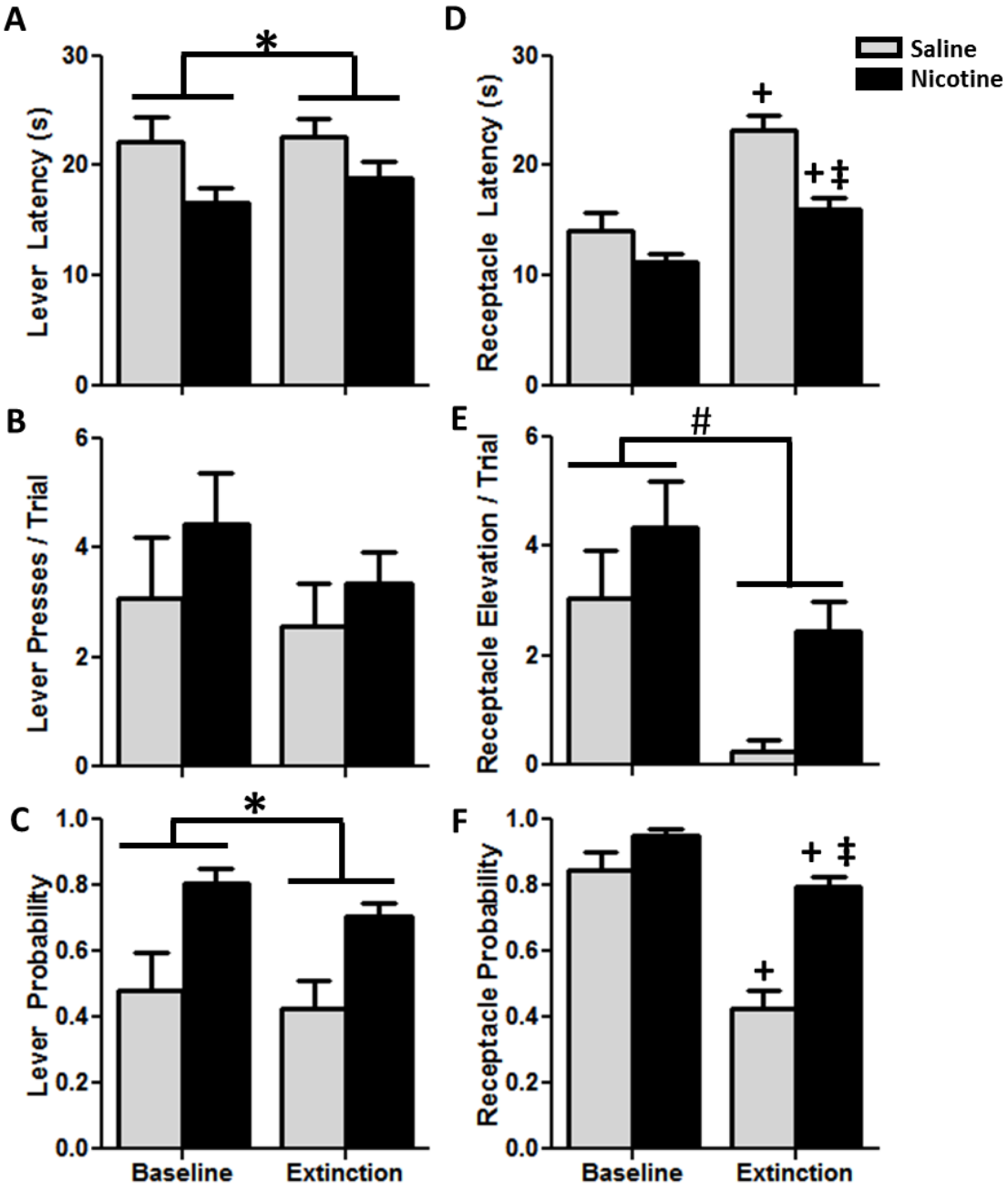

Figure 3.3: Withholding of expected sucrose reward reduces conditioned responding in NIC and SAL rats.

Behavioral measures were compared on a baseline day and the extinction test day in NIC and SAL groups of rats. Measures of sign tracking and goal tracking are as described in Fig1. * main effect of drug exposure, # main effect of water substitution day, + interaction, change in both drug groups from baseline to extinction day, ‡ interaction, difference between NIC and SAL only on the extinction day, $p<0.05$ for all analyses.

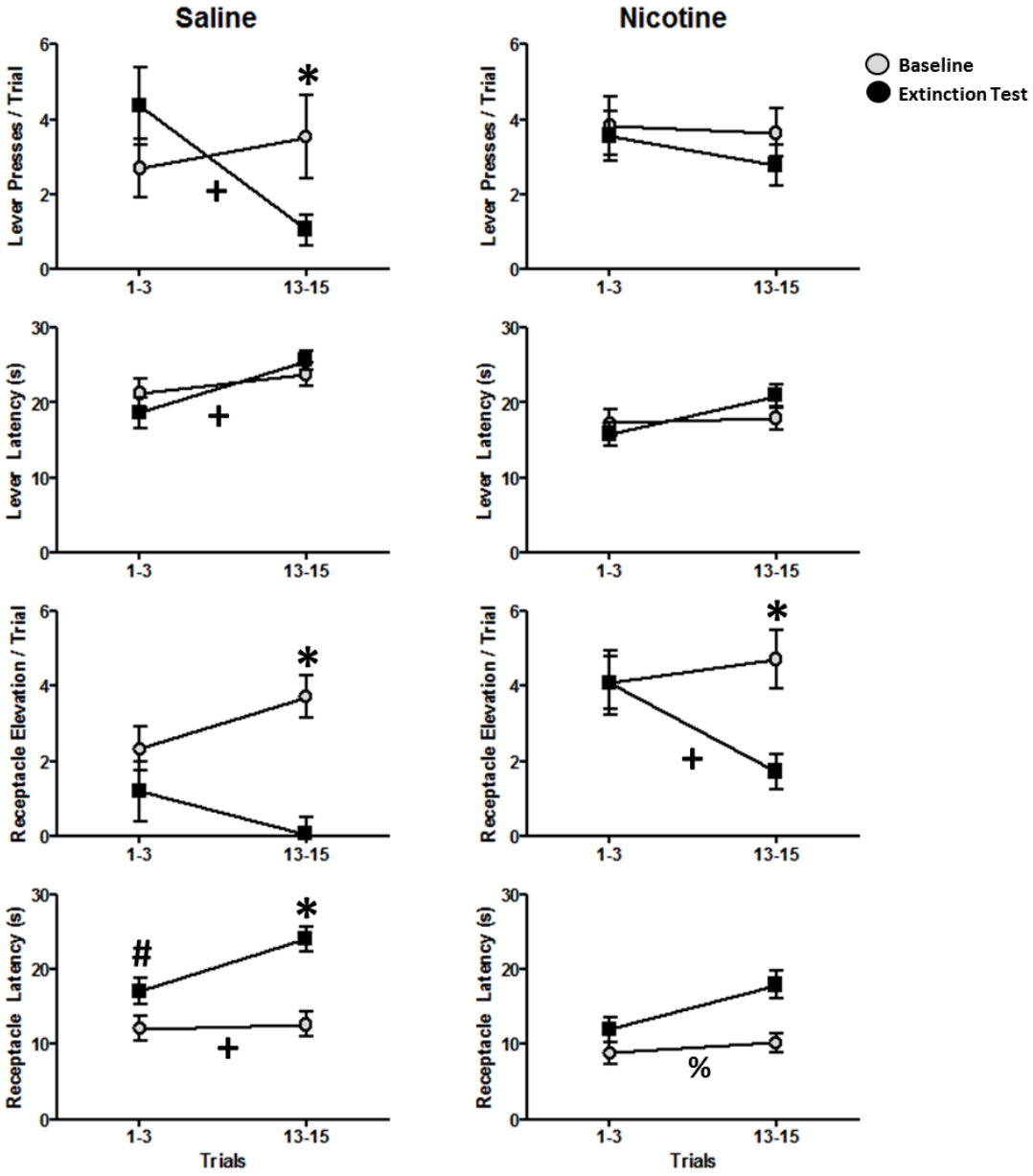

Figure 3.4: Withholding of reward reduces conditioned responding in NIC and SAL animals within the session

Conditioned responding during the baseline and extinction test sessions were compared within drug exposure group at the beginning of the session (Trials 1-3) and end of the session (Trials 13-15). Behavioral measures are as described in Fig 1. % main effect of extinction day, + interaction, difference between first and last trials on extinction substitution day, * interaction, difference between Trials 13-15 on the baseline and test days, # interaction, difference between trials 1-3 on baseline and extinction days, $p<0.05$ for all analyses.

	Dose	Saline	Nicotine
Receptacle Entries	Vehicle	5.9 ± 1.0	11.3 ± 3.0
	0.3mg/kg	5.5 ± 1.3	7.0 ± 1.3
	1.0mg/kg	5.0 ± 1.5	5.8 ± 1.1
	2.0mg/kg	5.2 ± 1.1	5.0 ± 1.1
	Slope	-0.3 ± 0.4	-2.6 ± 1.0*****
Receptacle Elevation	Vehicle	4.4 ± 1.1	8.1 ± 2.9
	0.3mg/kg	4.3 ± 1.3	4.9 ± 1.3
	1.0mg/kg	3.9 ± 1.4	3.5 ± 1.3
	2.0mg/kg	4.2 ± 1.0	2.9 ± 1.0
	Slope	-0.1 ± 0.4	-2.2 ± 1.0
Receptacle Latency	Vehicle	11.6 ± 1.8	7.3 ± 1.1
	0.3mg/kg	10.4 ± 1.0	9.2 ± 0.9
	1.0mg/kg	12.1 ± 2.9	9.8 ± 1.1
	2.0mg/kg	10.6 ± 1.5	11.7 ± 1.7
	Slope	0.2 ± 0.7	1.5 ± 0.4*****
Receptacle Probability	Vehicle	1.0 ± 0.0	1.0 ± 0.0
	0.3mg/kg	0.9 ± 0.0	1.0 ± 0.0
	1.0mg/kg	0.9 ± 0.1	1.0 ± 0.0
	2.0mg/kg	0.9 ± 0.1	0.9 ± 0.0
	Slope	0.0 ± 0.0	0.0 ± 0.0
Lever Presses	Vehicle	2.4 ± 1.0	6.4 ± 2.9
	0.3mg/kg	1.7 ± 0.7	4.4 ± 1.4
	1.0mg/kg	2.7 ± 1.2	4.9 ± 2.7
	2.0mg/kg	2.0 ± 1.0	4.6 ± 2.1
	Slope	0.0 ± 0.3	-0.6 ± 0.5
Lever Latency	Vehicle	19.8 ± 3.8	15.7 ± 3.3
	0.3mg/kg	20.2 ± 3.6	16.2 ± 1.9
	1.0mg/kg	18.1 ± 3.6	17.8 ± 2.1
	2.0mg/kg	20.3 ± 4.3	18.3 ± 2.7
	Slope	0.1 ± 0.7	1.3 ± 1.0
Lever Probability	Vehicle	0.5 ± 0.2	0.7 ± 0.1
	0.3mg/kg	0.5 ± 0.2	0.8 ± 0.1
	1.0mg/kg	0.6 ± 0.2	0.7 ± 0.1
	2.0mg/kg	0.4 ± 0.2	0.7 ± 0.1
	Slope	0.0 ± 0.0	-0.1 ± 0.1

Table 3.1. Naltrexone effects on conditioned approach in NIC and SAL animals.
Mean ± SEM behavioral responses for NIC and SAL animals, and average slope of the dose response curve over 3 doses of naltrexone and vehicle. * slope is significantly different from 0, $p<0.05$.

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Chapter 4: Sex differences in nicotine-enhanced Pavlovian conditioned approach

Introduction

An environmental stimulus that is repeatedly paired with a reward (unconditioned stimulus, US) can form a stimulus-outcome association through Pavlovian mechanisms. Exposure to this now conditioned stimulus (CS) can induce both humans and animals to display conditioned behavioral responses (Robinson and Berridge, 1993). In an animal model of Pavlovian conditioned approach, which allows for quantification of the conditioned responses (CR) that arise as a result of these Pavlovian associations, two behaviors typically emerge: animals can display the sign tracking CR, in which they approach and interact with the CS, or the goal tracking CR, in which they approach and interact with the location of eventual US delivery (Brown and Jenkins, 1968; Flagel et al., 2009).

Expression of the sign-tracking CR suggests that the CS itself has become an incentive stimulus, as it is able to attract attention and motivate approach (Flagel et al., 2007; Uslaner et al., 2006). In these animals, the CS also acquires conditioned reinforcing properties (Robinson et al., 2014). Animals that are categorized as sign trackers display enhanced drug self-administration and are more likely to show other behaviors associated with addiction vulnerability (Flagel et al., 2009; Lovic et al., 2011; Tomie et al., 1998). Rats bred to exhibit a high responding phenotype, in that they show increased locomotor response to novelty and impulsivity, are also likely to sign track (Flagel et al., 2016). Sign and goal trackers vary in the neurotransmitter and neuronal activation associated with a preferred conditioned response, suggesting that there are both neurobiological and genetic factors underlying the expression of these CRs (Campus et al., 2016; S. Flagel et al., 2011; Paolone et al., 2013; Saunders and Robinson, 2012; Singer et al., 2016).

Drug exposure has been shown to increase the expression of the sign-tracking phenotype, toward both drug-and non-drug-associated cues (Krank et al., 2008; Uslaner et al., 2006; Versaggi et al., 2016). Nicotine, in particular, exhibits reinforcement and incentive enhancing properties, suggesting that it amplifies the rewarding or incentive properties of non-nicotine stimuli (Chaudhri et al., 2007b; Palmatier et al., 2013b, 2007). We and others have shown that exposure to nicotine increases the likelihood that an animal will display an increase in either sign tracking alone (Guy and Fletcher, 2014a; Versaggi et al., 2016) or both sign- and goal-tracking in response to a nondrug associated CS (Palmatier et al., 2013b; Stringfield et al., 2017).

While investigating the effect of drugs such as nicotine on behavior, the potential for variation in responding due to sex should be considered. Most studies of Pavlovian conditioning paradigms that investigate both sign-and goal-tracking CRs use only male animals. When females have been included, moderate differences in conditioned responding emerge in that females are faster to acquire sign tracking and show more conditioned reinforcement of a lever CS (Madayag et al., 2017; Pitchers et al., 2015). In measures of conditioned US entries, females also show increased goal tracking (Hammerslag and Gulley, 2014). The influence of nicotine on sex differences in Pavlovian CR has yet to be established, although in animal models of nicotine self-administration, some studies indicate that females acquire self-administration faster than males (Donny et al., 2000; Lynch, 2009) while others find no difference between sexes (Feltenstein and See, 2008; Swalve et al., 2016). In terms of the relationship between nicotine exposure and the reinforcing properties of nicotine-associated cues, female rats respond more for nicotine reinforcement in the presence of a nicotine-paired stimulus than male animals (Chaudhri et al., 2005). In humans, female smokers are more sensitive to nicotine-associated stimuli than the pharmacological effects of nicotine while the opposite may be true in males (Perkins et al., 2002). Given this potential divergence between sexes in the influence of nicotine-associated cues, it follows to consider the influence of sex on

the incentive-amplifying properties of nicotine and the ability of nicotine exposure to influence nondrug-associated cues. Using a Pavlovian conditioned approach behavioral model, we can investigate the possibility that female animals exposed to nicotine will also show increased conditioned responding to a nondrug-associated stimulus compared to males.

Brain-derived neurotrophic factor (BDNF), a protein that modulates synaptic plasticity and is expressed throughout the central nervous system, has been associated with psychiatric disorders, behavioral responses, and drug abuse (Bath and Lee, 2006; Ghitza et al., 2010; Pitts et al., 2016). In humans, a single nucleotide polymorphism in the 66th nucleotide on chromosome 11 produces in a valine to methionine amino-acid substitution ($Val^{66}Met$) that results in reduced BDNF expression (Jamal et al., 2015; Notaras et al., 2015). Several studies have attempted to establish a link between the $Val^{66}Met$ polymorphism or serum BDNF levels and nicotine use, craving, and withdrawal (Bhang et al., 2010; Kim et al., 2007; Lang et al., 2007). For example, one recent study found that heavy smoking over long periods of time is correlated with increased BDNF protein expression, but was not correlated with the $Val^{66}Met$ polymorphism (Jamal et al., 2015). Another study identified expression of the Met allele as associated with increased smoking (Lang et al., 2007), but this relationship has yet to be replicated.

Preclinical studies in rodents have also aimed to elucidate the influence of BDNF expression in the brain on psychiatric disorders including drug addiction (Pitts et al., 2016; Wook Koo et al., 2016). Studies on nicotine indicate that chronic and acute exposure can modulate BDNF. Depending on the length of nicotine exposure, BDNF in the striatum and hippocampus can be increased (Kenny et al., 2000; Kivinummi et al., 2011) or reduced (Ortega et al., 2013; Yeom et al., 2005). Chronic exposure to a high dose of nicotine also results in deficits in reversal learning, and this reduction is correlated with reduced BDNF in the striatum (Ortega et al., 2013). Behavioral adaptations due to BDNF expression are also an area of interest, with some studies finding that knockdown of BDNF in the prefrontal cortex in genetic mouse models

influences both conditioned place preference and habitual behavior (Gourley et al., 2016; Zimmermann et al., 2015). In drug-naïve sign-and goal-trackers, sign trackers were reported to have reduced BDNF in the prefrontal cortex, but not the BLA or striatum (Morrow et al., 2015). Thus, BDNF expression in key corticolimbic brain regions appears to be modified after drug exposure, and this altered expression may influence reward-associated behaviors.

We and others have previously shown that nicotine exposure increases Pavlovian conditioned approach, and in this study, we continue to explore this intersection between nicotine and individual variability by evaluating the effect of nicotine on BDNF protein expression in key brain regions associated with reward and motivation in male and female rats. We hypothesize that nicotine exposure will increase the expression of conditioned responding in both sexes, but females may show elevated conditioned approach compared to males. In addition, we hypothesize that BDNF expression will be enhanced in animals exposed to nicotine compared to control animals.

Methods

Animals

A total of 24 male and 24 female Sprague Dawley rats (225-250g males, 174-190g females on arrival) were purchased from Harlan/Envigo (Indianapolis, IN, USA). Animals were housed in same-sex pairs in a vivarium on a 12:12 hour light:dark cycle, and experiments were run during the light cycle. Throughout the experiment, rats were provided with food and water *ad libitum*. This experiment was conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Behavioral training

Behavioral training occurred during Pavlovian conditioning sessions described previously (Stringfield et al., 2017). Animals were exposed to the 20% sucrose (w/v) solution

that would be used as the US for 1 hour. Rats were then assigned to a nicotine (NIC) or saline (SAL) drug exposure group, and received a single injection of the assigned drug on two consecutive days to habituate the animals to the stress of injections. Nicotine hydrogen tartrate salt (Sigma-Aldrich, St. Louis, MO), dissolved in sterile saline with the pH adjusted to 7.0 ± 0.2, was injected at a dose of 0.4 mg/kg (s.c., calculated using the free base form). SAL animals received an equivalent volume of saline.

Prior to Pavlovian conditioning sessions, animals were introduced to the testing chambers during a magazine training session. For this session, rats were injected with the assigned solution 10 minutes before being placed into a plexiglass operant chamber (MedAssociates, St Albans, VT). Each chamber was assembled with a stimulus light, retractable lever, and recessed reward receptacle on one wall of the chamber and a house light on the opposite wall. Animals remained in the testing chamber for 5 minutes before session initiation. During this session, the house light was illuminated throughout the session and animals received 15 deliveries of 0.1ml of the 20% sucrose US into the reward receptacle on a VI120s schedule of reinforcement. Head entries into the receptacle were recorded by a photobeam detector but had no programmed consequences.

After magazine training, all animals underwent 29 Pavlovian conditioning sessions. These sessions were initiated as described above, with injections occurring 15 minutes before the start. Each session consisted of 15 trials during which the CS (extension of the retractable lever and illumination of the stimulus light) was presented for 30s on a VI120s schedule. Animals were able to interact with the lever during cue presentation, and both lever presses and receptacle entries were recorded, but had no programmed consequences. After the CS presentation, the stimulus light extinguished, the lever retracted, and 0.1ml of the 20% sucrose US solution was delivered into the receptacle.

Tissue processing

Approximately 24 hours after the final Pavlovian conditioning session, animals were euthanized and brains were collected for western blotting. Animals were rapidly decapitated without anesthesia by trained personnel and brains were removed and flash frozen using isopentane cooled with dry ice. Brains were stored at -80°C before processing. Tissue punches of each region of interest were taken from coronal sections on a cryostat using a 1mm tissue punch (Miltex, York, PA). Samples were diluted in homogenization buffer [1% sodium dodecyl sulfate (SDS), 10mM Tris (pH 7.4), protease inhibitor cocktail tablets (Roche, Indianapolis, IN)], homogenized using a sonicator probe, and then centrifuged at 4°C for 15 minutes at 12,000×*g*. Protein concentration of the supernatant was determined using the Pierce BCA assay (Thermo Fisher, Waltham, MA).

Western blot procedure

Twenty micrograms of protein was diluted in Laemelli sample buffer (Bio-Rad Hercules, CA) and boiled at 95°C for 5 minutes before being loaded on a precast 4-15% Tris-glycine gel (Bio-Rad) for SDS-polyacrylamide gel electrophoresis in Tris/glycine/SDS running buffer. Proteins were transferred to a polyvinylidene fluoride membrane using the Trans-Blot Turbo Blotting System (Bio-Rad) with transfer settings for mixed molecular weight proteins. Membranes were blocked in blocking solution containing 5% nonfat milk in tris-buffered saline with 0.1% Tween 20 (TBST) for 1 hour at room temperature, and then incubated at 4°C overnight with primary antibodies against BDNF [ab108319 rabbit anti-BDNF, 1:1000 (Abcam, Cambridge, MA)] or GAPDH [MA5-15738 mouse anti-GAPDH, 1:1000 (Thermo Fisher)] in 1% blocking solution. Membranes were washed in TBST and then incubated with secondary antibodies (HRP-conjugated donkey anti-rabbit or goat anti-mouse, 1:5000) in 1% blocking solution for 2 hours at room temperature. Enhanced chemiluminescence substrate (Bio-Rad) was added and blots were imaged using the ChemiDoc Imaging System (Bio-Rad). Bands for

the mature form of BDNF were visible at 15kda and GAPDH at 37kda. Quantification of band intensities was completed using Bio-Rad Image Lab software.

Statistical analysis

Analysis of behavioral responses by male and female animals was completed using SigmaPlot v11.0 software (SyStat Software Inc, San Jose CA). The last 4 days of training were averaged and compared between male and female rats exposed to nicotine or saline by 2- way ANOVA followed by Tukey's HSD for post hoc comparisons. Behavioral measures analyzed were latency to press the lever or enter the reward receptacle during the 30 s cue presentation, lever presses per trial, a receptacle elevation score, and the probability of entering the receptacle or pressing the lever during a trial. Elevation scores were calculated by subtracting the number of receptacle entries that occurred during a 30 s period before a trial began from the number of receptacle entries that occurred during a 30 s trial (Palmatier et al., 2013b; Stringfield et al., 2017). The probability of a lever press or receptacle entry was calculated as the number of trials in which the behavior occurred, divided by the total number of trials in a session. α was set at 0.05 for all analyses and corrected for multiple comparisons as appropriate. To compare day-to-day variability between sexes, a coefficient of variation was calculated for each of the above behavioral measures for each rat across the last 4 days of training (Guizzetti et al., 2016; Madayag et al., 2017), and analyzed by 2-way ANOVA. In addition, we calculated a Pavlovian conditioning score to categorize animals as goal trackers, sign trackers, or intermediate animals that takes into account the above-mentioned measures of conditioned responding (Madayag et al., 2017). The formula for this tracking score is as follows:

$$\frac{\left(\frac{lev.press.-elev.score}{lev.press.+abs.value\ of\ elev.score}\right)+\left(\frac{recept.latency-lev.latency}{30}\right)+\left(\frac{CS\ trials-US\ trials}{CS\ trials+US\ trials}\right)}{3}$$

BDNF protein expression as detected by western blots was normalized to GAPDH loading control. BDNF expression in male and female NIC and SAL animals was compared by 2-way ANOVA, both using the raw BDNF/GAPDH ratio and after normalizing to SAL female

controls within each blot. Protein expression in NIC-exposed male and female animals was expressed as a percent change from saline controls and analyzed by independent samples t-test. In addition, to investigate the relationship of frontostriatal BDNF expression to behavior, we calculated a ratio of cortical /striatal BDNF for all animals (Parikh et al., 2016) and correlated this ratio with behavioral measures within each sex or drug exposure group using Pearson's correlation coefficient.

Results

Pavlovian conditioned approach behavior

Both male and female animals successfully formed an association between CS presentation and subsequent US delivery over time regardless of drug exposure, as shown by an increase in conditioned responding during the 29 days of training in all groups (Figures 1 and 2). Nicotine exposure enhanced both sign-and goal-tracking conditioned responses, and females showed slightly elevated sign tracking behaviors on some measures. For sign tracking behaviors (Figure 1), there was a main effect of nicotine exposure on lever presses ($F_{1,47}$ =4.1, $p<0.05$), latency to press the lever ($F_{1,47}$ = 8.5, $p<0.01$), and probability of pressing the lever ($F_{1,47}$ = 6.2, $p<0.05$). No difference between male and female animals emerged for lever presses, but there was a trend toward a main effect of sex on lever latency ($F_{1,47}$ = 3.9, $p=0.054$) with females showing a reduced latency, and a main effect of sex emerged for lever probability ($F_{1,47}$ =4.1, $p<0.05$) in which females were more likely to press the lever. Nicotine exposure also enhanced expression of goal tracking behaviors in both male and female animals (Figure 2), but no effect of sex emerged. Nicotine exposure increased receptacle elevation score ($F_{1,47}$ = 4.9, $p<0.05$) and probability of entering the receptacle ($F_{1,47}$ = 4.3, $p<0.05$) but did not influence the latency to enter the receptacle ($F_{1,47}$ = 2.6, $p>0.05$).

In addition to looking at sex and drug exposure differences on separate measures of conditioned responding, we also computed a Pavlovian conditioned approach score to

categorize animals as sign or goal trackers (Table 1). Males were more likely to be classified as goal trackers than females, and nicotine exposure reduced the number of goal trackers within both sexes.

If estrous cycle influenced conditioned approach, it follows that females would show increased day-to-day variability than males. To assess individual variability by sex, we calculated the coefficient of variation (CV) across the last 4 days of training for male and female rats in both the NIC and SAL groups (Table 2). There was a sex × drug exposure interaction for receptacle probability ($F_{1,44} = 4.2$, $p<0.05$), in that within females, SAL rats had a higher CV than NIC rats. The only behavioral measure that showed a main effect of sex was receptacle latency ($F_{1, 47} = 8.3$, $p<0.05$) where females showed a higher CV than males. In addition, a main effect of drug exposure emerged for lever latency ($F_{1,47} = 6.8$, $p<0.05$) in which NIC animals had a higher CV than SAL rats.

Western immunoblot for BDNF

Tissue from the OFC, NAc, and BLA was analyzed by western blot to measure expression of BDNF protein. The mature form of BDNF protein was measured at 15kda. BDNF protein was normalized to GAPDH protein levels for all analysis. Analysis of BDNF/GAPDH ratios, both raw and normalized to SAL females, yielded no significant results in any region ($p>0.05$ for all analyses). BDNF protein expression in males and females was expressed as a percent of saline control in each region, to assess the effect of nicotine exposure (Figure 3). While there was a difference in mean protein expression after nicotine exposure, no significant differences emerged ($p>0.05$). Next, BDNF protein expression was correlated with behavioral responses in all 3 regions tested. While BDNF protein expression was more likely to be correlated with behavior in NIC exposed animals regardless of sex, none of the correlations reached statistical significance (data not shown). Finally, a ratio of BDNF protein expression in the OFC and NAc was developed to assess potential biasing of frontostriatal synaptic plasticity

in NIC animals, similar to previous studies (Parikh et al., 2016) and correlated to behavioral responses (Figure 4). Here, a significant correlation emerged in female NIC animals for lever latency (Pearson's $r = 0.59$, $p<0.05$), but no other correlations for OFC/NAc BDNF ratio, OFC/BLA ratio, or BLA/NAc ratio emerged.

Discussion

In this study, we replicated our previous findings that nicotine enhances conditioned approach in males and extended this result to females. We found some difference in behavior between females and males, primarily in the extent of nicotine-enhancement of sign tracking conditioned responses. In addition, females as a whole were less likely to be classified as goal trackers compared to males. We evaluated BDNF protein expression in the same animals, and found no significant differences between sexes or by nicotine exposure in the OFC, NAc, or BLA. However, when we created a ratio of BDNF protein in the OFC / NAc protein expression, we did see a correlation with latency to press the lever specifically in NIC females. This supports the observed sex difference in behavior, but indicates that BDNF expression may not be strongly associated with other aspects of conditioned approach or nicotine exposure in general.

The mild sex difference in conditioned responding described here fits with previous studies of sign-and goal-tracking animals, suggesting that sex does not necessarily contribute a large amount of variability to this behavior (Pitchers et al., 2015). Our lab has recently observed that female rats showed more sign tracking behavior and were more likely to be classified as sign trackers in a study that investigated conditioned approach after adolescent intermittent ethanol exposure (Madayag et al., 2017). In this previous study, rats were bred in house instead of purchased from a vendor, which may explain the difference in the magnitude of the observed effects in females. These results support the importance of inclusion of both male and female animals when measuring behavior (Becker et al., 2016; Guizzetti et al., 2016). In some cases, drug exposure can influence females differently than males, even if there were no preexisting

differences due to sex. Nicotine has been suggested to influence both female and males differently in preclinical and clinical populations (Perkins et al., 1999; Pogun and Yararbas, 2009). Female rodents are more sensitive to nicotine associated stimuli, while males are more sensitive to the subjective effects of nicotine (Chaudhri et al., 2005), and studies in male and female human smokers have supported this possibility (Perkins et al., 2000). This slight difference in enhanced sensitivity to conditioned cues may explain the elevated sign tracking behavior observed in the present experiment. Of note for this study, sex differences in the locomotor activating effects of nicotine may not be present in rodents (Isiegas et al., 2009; Kanýt et al., 1999), suggesting that the decreased latencies seen in females is primarily due to the incentive motivational properties attributed to the salient CS, and not due to sex-specific hyperactivity.

While we did not directly measure estrous cycle phase to be able to draw conclusions about the effects on behavior, Pitchers and colleagues (2015) previously demonstrated that estrous cycle does not contribute additional variability to the sign-and goal-tracking behavior in females. To attempt to evaluate potential variability in behavior across the estrous cycle, we analyzed the coefficient of variation over the last 4 days of training. We found that for the majority of behavioral measures, there was no difference in variability in female rats compared to males, replicating out previous study. There was a difference in variability for receptacle latency, but in analysis of group differences on this behavioral response, there were no significant differences between males and females or NIC and SAL exposure groups. Thus, it does not appear that hormonal variations across the estrous cycle contributed to the sex differences that did reach significance.

We found no difference in BDNF protein expression as measured by western blot between male and female rats, after nicotine exposure, or when correlated with behavioral response. Although others have found differences in BDNF expression based on sign-or goal-tracker classification (Morrow et al., 2015), in which sign trackers had reduced BDNF

expression in the prefrontal cortex, we did not see such effect in the OFC, NAc, or BLA. When we analyzed BDNF expression as a ratio of protein in the OFC related to the NAc, we found a correlation with latency to press the lever specifically in NIC females. This was one behavioral measure where we identified an effect of sex on conditioned responding, and the additional correlation with BDNF suggests that the protein exhibits slight changes in expression based on sex, nicotine exposure, and behavior. Others have demonstrated that a ratio of cortico-striatal BDNF, calculated based on protein expression in the dorsal striatum and PFC, is correlated with perseverative errors during nicotine withdrawal (Parikh et al., 2016). In a separate study, BDNF expression in the PFC was correlated with instrumental responding for a food reward, while expression in the striatum was not (Gourley et al., 2016). These results suggest that the balance of BDNF expression in striatum and prefrontal cortex is relevant to the expression of multiple behaviors and warrants continued study.

We hypothesized that differences in OFC BDNF levels would be apparent preferentially in nicotine exposed animals, given that others have shown both increases and decreases in corticolimbic BDNF after acute or chronic nicotine exposure (Kivinummi et al., 2011; Ortega et al., 2013; Yeom et al., 2005). These previous studies utilized alternate nicotine exposure regimens resulting in higher doses of nicotine over either the acute period, or after a chronic nicotine regimen that stimulates persistent changes in behavior and synaptic plasticity. We have shown that in our paradigm and using the current dose of nicotine, withholding nicotine injections before a training session resulted in a reduction in behavior that returned the NIC-enhanced conditioned responses to the level of control animals (Stringfield et al., 2017). The 24-hour time point was chosen to avoid measuring acute effects of nicotine exposure on BDNF protein, and instead to measure longer lasting effects of multiple days of drug exposure. It is possible that the nicotine dose utilized in this study was not high enough to elicit changes in BDNF expression that lasted for 24 hours.

Similarly, it is possible that the 24-hour time point chosen for tissue collection obscured any changes in BDNF expression that may have occurred. These animals had been trained for 29 days prior to tissue collection, and no changes to the Pavlovian training sessions had occurred during this time. BDNF is involved in in synaptic plasticity as well as learning and memory (Jia et al., 2010; Leal et al., 2014; Tyler et al., 2002), and differences in expression may have occurred at the time of initial acquisition of the conditioned approach behavior. A future test of BDNF protein expression related to behavior and nicotine exposure could incorporate new learning, such as extinction training or a reversal task, to challenge the ability of sign-and goal-tracking rats to update their behavior after a change in stimulus-outcome contingency.

A possible limitation to this study is the use of western blotting for protein detection. Other studies that have correlated behavioral responses to BDNF expression have utilized ELISA to measure BDNF and found differences in protein in the hippocampus or corticolimbic regions related to conditioned approach response or nicotine exposure (Morrow et al., 2015; Ortega et al., 2013; Parikh et al., 2016). The use of western blotting allowed for measurement of specific isoforms of BDNF protein based on molecular weight, as both pro-BDNF and mature BDNF can be biologically active (Lipsky and Marini, 2007; Park and Poo, 2013; Pitts et al., 2016). As other studies have identified variation in total BDNF protein expression by ELISA, it is possible that differences were present in other BDNF isoforms in the present study. However, BDNF protein expression in the targeted areas of the brain, particularly the NAc and BLA, is relatively low due to the primary dependence on secreted BDNF from efferent projections into these areas (Conner et al., 1997). In this case, the increased sensitivity afforded by an ELISA may be more likely to register minute differences in BDNF expression. Replication of this study using an ELISA could provide additional information about changes in total BDNF by detecting smaller variations in protein expression, but further analysis would need to be conducted to determine if any change in total BDNF is driven by specific isoforms of the protein.

This study presented evidence that nicotine enhances conditioned approach in both males and females. In addition, we add to the growing literature of sex differences in reward conditioning, in that females show some increase in the likelihood to express the sign tracking response compared to males. BDNF protein in mesocorticolimbic brain regions proposed to be involved in this behavior was not influenced by sex, conditioned responding, or NIC exposure, although the potential importance of the balance between cortical-striatal BDNF warrants further study.

Figures

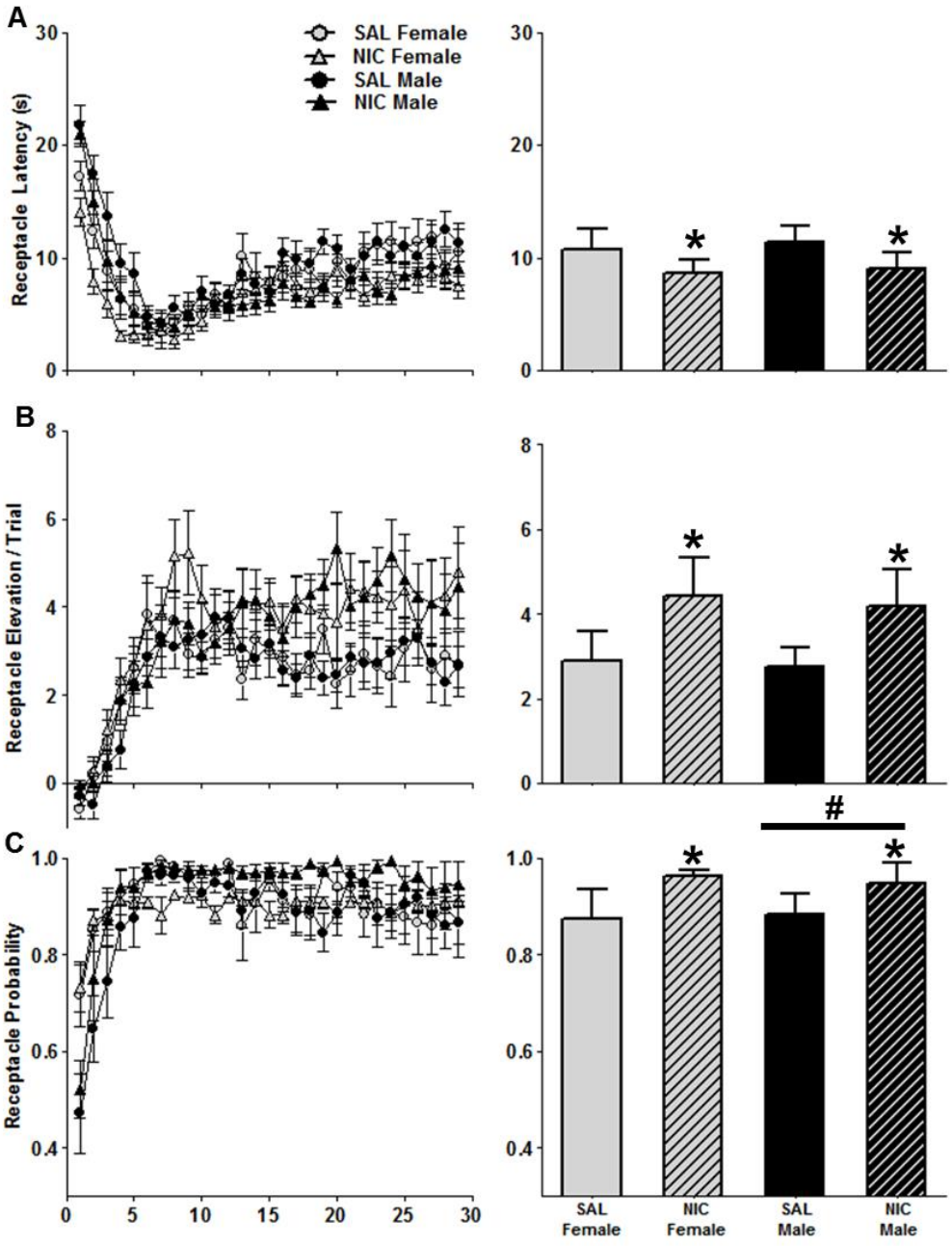

Figure 4.1: Nicotine enhances sign tracking in male and female rats.
Expression of sign-tracking behaviors over 29 days of training (left) and averaged across the last 4 days of training (right) in male and female rats that received nicotine injections prior to training, or in the saline control group. Data are expressed as mean ± SEM, and reflect separate measures of conditioned approach behavior. (A) Latency to press the lever, (B) lever presses per trial (C) probability of pressing the lever. * Main effect of nicotine exposure, # main effect of sex, $p<0.05$.

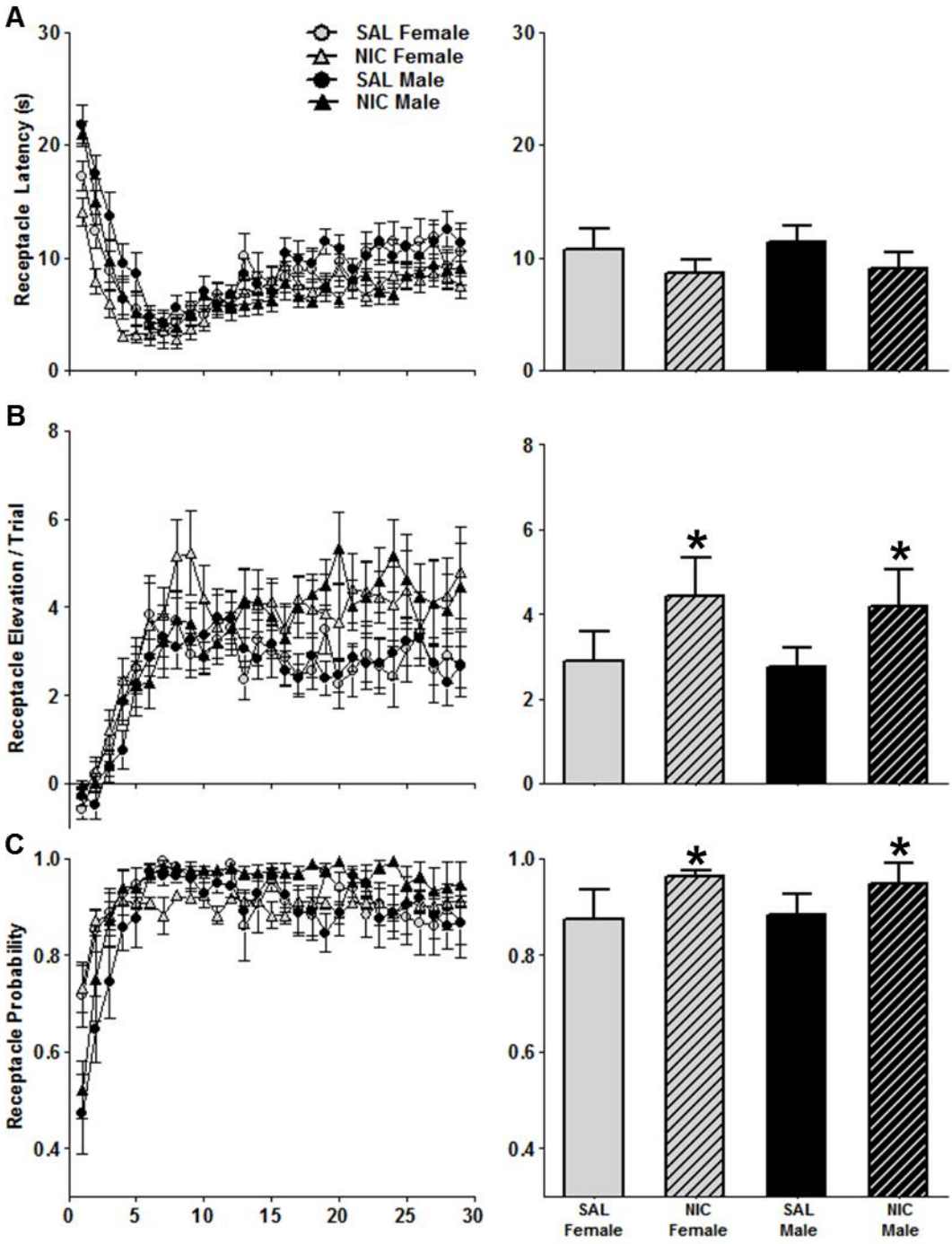

Figure 4.2: Nicotine enhances goal tracking in male and female rats.
Expression of goal-tracking behaviors over 29 days of training (left) and averaged across the last 4 days of training (right) in male and female rats exposed nicotine or in the saline control group. Data are expressed as mean ± SEM. (A) Latency to enter the receptacle, (B) receptacle elevation score per trial (C) probability of entering the receptacle. * Main effect of nicotine exposure, $p<0.05$.

	Goal Tracker	Intermediate	Sign Tracker
SAL Female	25%	50%	25%
SAL Male	46%	31%	23%
NIC Female	0%	58%	42%
NIC Male	9%	82%	9%

Table 4.1. Distribution of sign and goal tracking animals by sex and drug exposure
A tracking score was calculated for each rat based on conditioned approach behavior on the last 4 days of training. The score was used to classify rats within sex and drug exposure group as goal trackers, intermediate, or sign trackers.

	Lever Latency[c]	Lever Press	Lever Probability	Receptacle Latency[b]	Receptacle Entries	Receptacle Probability
SAL Female	19.2 ± 2.8	36.5 ± 10.9	14.6 ± 9.7	28.7 ± 3.2	30.1 ± 3.9	12.6 ± 2.7[a]
SAL Male	13.5 ± 2.7	45.7 ± 10.5	28.1 ± 9.4	23.7 ± 3.1	23.7 ± 3.7	1.2 ± 2.7
NIC Female	22.4 ± 2.8	16.0 ± 10.9	2.6 ± 9.7	28.6 ± 3.2	22.8 ± 3.9	6.4 ± 2.6[a]
NIC Male	24.0 ± 3.0	42.2 ± 11.4	25.4 ± 10.1	15.1 ± 3.4	16.0 ± 4.1	6.3 ± 2.9

Table 4.2. Individual variability in behavior by sex and drug exposure across last four days of training.
The coefficient of variation was calculated for each animal and averaged across groups for each sign-or goal-tracking behavior. Data are presented as mean ±SEM, a: sex × treatment interaction with a difference in receptacle probability in females in the SAL group, b: main effect of sex, c: main effect of treatment.

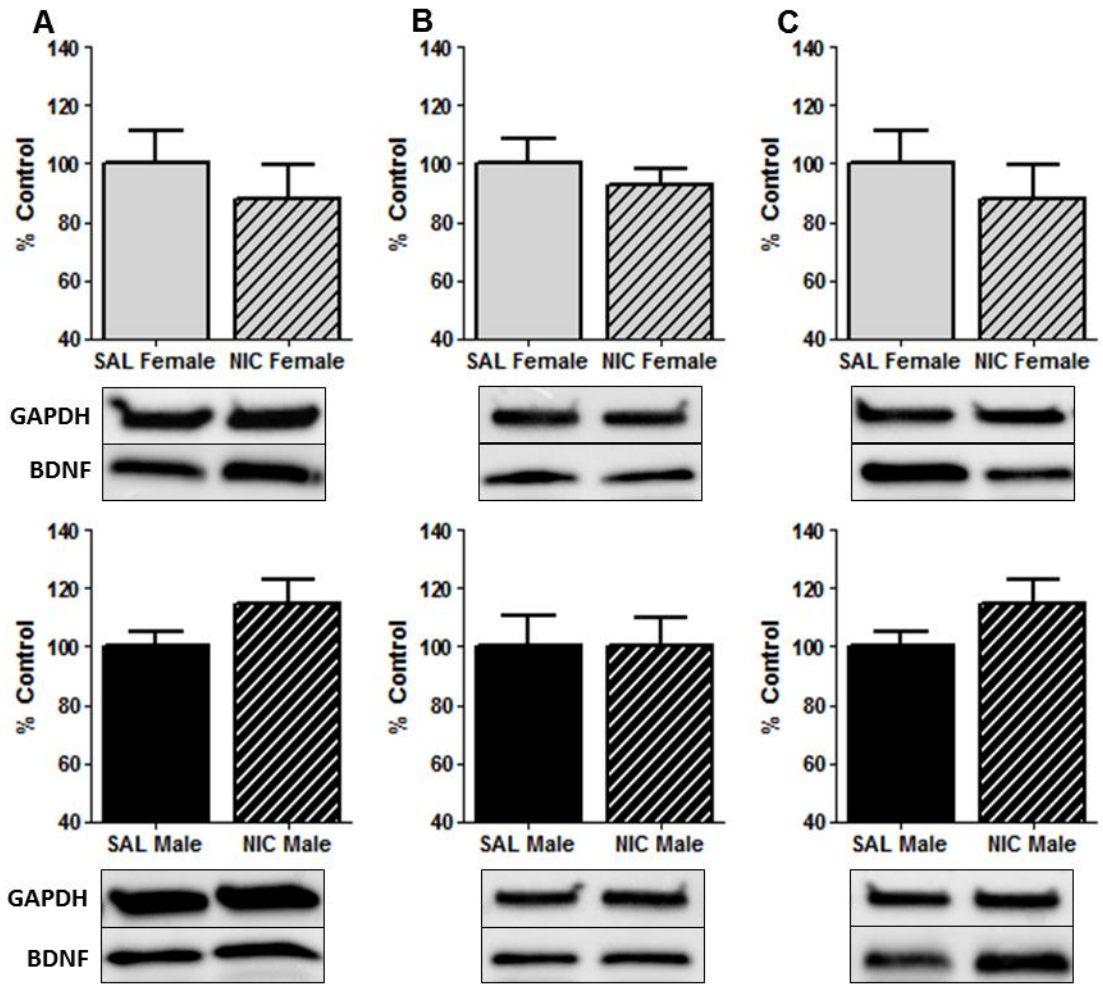

Figure 4.3: BDNF protein expression in the OFC, NAc, or BLA was not significantly influenced by sex or drug exposure.
BDNF protein expression was normalized to GAPDH loading control, and expressed as a % of saline control for males and females. BDNF expression was measured in the (A) OFC (B) NAc and (C) BLA in females (top) and males (bottom). Representative bands of GAPDH and BDNF protein are presented for each group.

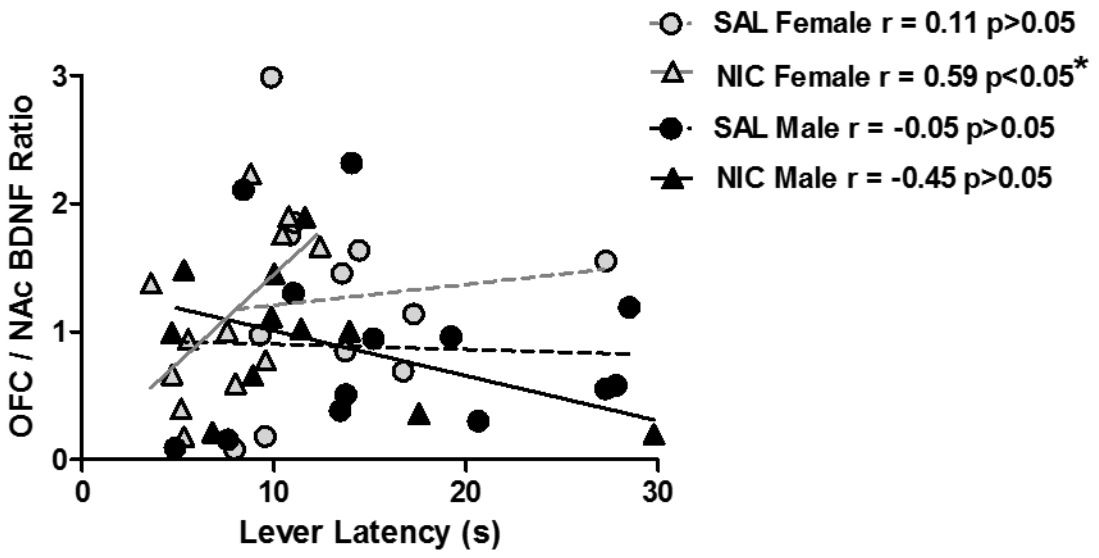

Figure 4.4: OFC/NAc BNDF ratio is correlated with lever latency in nicotine-exposed females.
A ratio of BDNF protein expression in the OFC and NAc was created and correlated to latency to press the lever for male and female rats in the NIC and SAL groups using Pearson's product moment correlation. * Significant correlation, $p<0.05$.

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Chapter 5: Discussion

Summary of findings

The experiments within this dissertation were designed to evaluate the influence of nicotine on the expression of Pavlovian conditioned approach and to advance our understanding of the circuit that is involved in this behavior. We hypothesized that nicotine, due to its ability to enhance the reinforcing or incentive properties of a stimulus, would also enhance sign-tracking approach behavior which is thought to signify the attribution of motivational properties to a conditioned cue. We also hypothesized that the stimulus-outcome association would be represented by the OFC while rats were demonstrating that the cue had, in fact, acquired salience.

We found that nicotine increased the expression of both the sign- and goal-tracking conditioned responses, consistent with other reports of nicotine-induced enhancement of approach behavior (Palmatier et al., 2013b; Yager and Robinson, 2015). This enhanced conditioned approach was resistant to manipulation of the expected outcome. Animals were less likely to change their sign-tracking behavior regardless of drug treatment, and NIC rats exhibited reduced updating of goal tracking as well. The orbitofrontal cortex was involved in representing receptacle entries, where goal tracking receptacle entries and approach to the goal cup to retrieve the reward engaged the most phasic firing in the OFC. Nicotine acutely blunted this increase in firing rate, but only when the drug was on board during the test session. We confirmed that nicotine elevates sign-and goal-tracking behaviors in females as well as males, which contributes substantially to the literature on this behavior as the majority of studies of Pavlovian conditioned approach, with or without nicotine, have been conducted using males. We identified a sex difference in the expression of conditioned approach, in that females

showed more sign tracking on some measures, and were more likely to be classified as sign trackers than males. This agrees with previous results from our lab (Madayag et al., 2017) as well as evidence from human imaging studies indicating that females may be more sensitive to the nonpharmacological components of addiction and nicotine, such as conditioned stimuli, compared to males (Rose, 2006). Finally, we investigated the expression of BDNF protein in the OFC, BLA, and NAC, which did not correlate directly with nicotine exposure or conditioned approach behavior, but the ratio of BDNF in the OFC and NAc did correlate with latency to press the lever in NIC females. These results suggest the need to replicate the experiment using a more sensitive measure of BDNF to identify possible small changes in BDNF protein expression, or confirm this null result.

General discussion

In Chapter 2, we reported that nicotine exposure resulted in the elevation of conditioned approach in male rats, and we replicated this effect and extended it to female rats in Chapter 4. Surprisingly, when nicotine was not administered immediately before the session, conditioned approach responses as well as OFC firing rates were returned to control levels. It was expected that since the Pavlovian stimulus-outcome association had been learned in the presence of nicotine, and was always expressed in the presence of nicotine, the enhanced salience of the CS would continue to motivate behavior in the absence of the drug. A previous study by Palmatier and colleagues (2013) using the same dose of nicotine and similar behavioral paradigm found that animals continued to express high levels of sign tracking in the absence of nicotine for 24 days. In humans, smoking cues are capable of eliciting craving during abstinence or withdrawal (McClernon et al., 2008), suggesting the incentive amplifying effects of nicotine on the acquired salience of a conditioned cue should be long lasting. Other studies have found that rats that show elevated Pavlovian approach due to nicotine exposure require nicotine to be pharmacologically active to show enhanced conditioned reinforcement (Guy and Fletcher, 2014b, 2013), and when tested in the absence of nicotine, these rats respond at the same level

as controls. Thus, it does appear that a conditioned association learned in the presence of nicotine should be maintained when the drug is no longer biologically active, but the ability of nicotine to enhance the incentive properties of a cue does not translate to new behaviors learned in the absence of nicotine. It is possible that in our study, the lack of nicotine during training was interpreted by the rats as a contextual change, given that the rats could have been anticipating the conditioned locomotor and interoceptive effects of nicotine. The absence of these effects may have resulted in a decrease in behavior. Extending the test of conditioned approach in the absence of nicotine for multiple days would confirm that the change in behavior that we measured was not a result of animals' conditioned expectation of the pharmacological effects of nicotine.

In Chapter 2, we found that inactivation of the OFC by infusion of the $GABA_A$ and $GABA_B$ agonists baclofen and muscimol reduced sign tracking regardless of drug exposure, but only reduced goal tracking in NIC animals on one measure of behavior. In contrast, when we recorded neurons from the OFC, we found a greater representation of goal-tracking behaviors, in the form of receptacle entries, in both nicotine and saline groups. Neurons in the OFC were not phasically active surrounding sign tracking (i.e., lever presses) to the same extent. The reduction in sign tracking after inactivation suggests that the OFC does play a role in this conditioned response, even if it is not directly encoding approach and interaction with the CS. It is possible that the role of the OFC is more nebulous when it comes to the actual expression of sign tracking, and that other areas of the brain previously shown to be directly involved in encoding the approach response such as the NAc (Day et al., 2006) are responsible for driving the behavior. Instead, the OFC may be responsible for the representation of the incentive value of the CS. This hypothesis is similar to the theory proposed by Wilson and colleagues (2014) that the OFC holds a representation of the current state of the task, such as the presence or absence of the conditioned cue along and its current value. Perhaps by inactivating the OFC

and blocking access to this representation, rats were less motivated to approach the CS. The finding that inactivation only reduced goal tracking in nicotine animals is also surprising, as OFC neurons fire specifically to goal-tracking behavior. This result confirms earlier reports following post-training lesions of the OFC, where Pavlovian conditioned approach to the US was not affected if the lesions were made after the stimulus-outcome association had already been learned (Chudasama and Robbins, 2003). The fact that the change in behavior only occurred in NIC animals would need to be replicated using a different method for OFC inactivation, as the potential for interaction of nicotine with the GABAergic agonists may confound these results. Nicotine influences GABAergic transmission in the PFC (Feduccia et al., 2012), and further increasing GABAergic agonism in the OFC potentially muddles the drug effect. These results could be replicated with a form of inactivation that should not be influenced by the pharmacological effects of nicotine, such as use of chemogenetic tools to transiently inactivate the OFC. A chemogenetic approach to investigating this question would also be desirable as it would allow for targeting glutamatergic projection neurons within the OFC, allowing for a more nuanced investigation of the contributions of the OFC to this circuit.

The finding that OFC neurons were not phasically active to the same extent surrounding a lever press compared to receptacle entries is interesting, given that this differs from studies of operant tasks which demonstrate that the OFC fires surrounding the action of pressing a lever (Moorman and Aston-Jones, 2014). This dichotomy between firing around a lever press does fit with a proposed function of the OFC, in encoding actions that are tied to receipt of reward or reward-seeking (Moorman and Aston-Jones, 2014; Schoenbaum et al., 2009). Lever pressing during an operant task is expected to produce a reward, but during the sign-tracking behavior, a lever press has never been causally linked with the expectation of reward delivery or receipt of a reward. Receptacle entries, on the other hand, have been associated with acquiring the reward. Even though the animal is aware that no reward should be present, the action of nose poking

into the reward receptacle is still tied with reward retrieval, and OFC neurons encode this action. This result verifies other findings of increased phasic firing surrounding a receptacle entry that is likely to be rewarded seen during other tasks (Moorman and Aston-Jones, 2014), as in this experiment the OFC fired more during receptacle entries in which the cue was present, than during entries that occurred outside of the cue presentation.

Taking these results together, we can draw some conclusions about the function of the OFC in situations where appetitive approach behaviors have already been learned and no new learning is taking place. It appears that the OFC is involved in expression of sign tracking, but does not encode in its neuronal firing pattern the action of approach to the CS and pressing the lever. Conversely, neurons in the OFC encode approach responses that are tied to reward retrieval, but the OFC is not required for the expression of the approach response once it has already been learned.

One follow up-study to further probe the role of the OFC in representing the salience of a conditioned cue would be to record from the OFC during a test of conditioned reinforcement, where the animals have to perform an operant response for presentation of the previously trained Pavlovian CS. The OFC could be responsible for encoding this type of operant response as it would be related to reward acquisition, similar to goal-tracking receptacle entries. This would differ from sucrose-seeking, however, as this behavior requires that the CS itself has become a reinforcer. The involvement of the OFC in representing the incentive value of a conditioned cue has been implied, as the OFC shows a pattern of immediate early gene activation that differs between sign trackers and goal trackers (S B Flagel et al., 2011), and extracellular recordings from the OFC suggest that certain populations of neurons encode the salience of an appetitive cue (Schoenbaum et al., 2003), but no study has directly demonstrated that the OFC specifically encodes this property of an incentive CS.

We also recorded neurons that were phasically active to cue presentation. This result supports the numerous studies in humans that show increased activation of the OFC in response to cues that predict a reward, and that the OFC is active in response to smoking cues (Childress et al., 1993; Gottfried et al., 2003a; McClernon et al., 2008). The result that nicotine blunted firing in the OFC compared to controls appears to oppose findings in humans that indicate that nicotine enhances neuronal activation to nicotine-associated cues; however, this study did not measure cues that were explicitly tied to the rewarding properties of the drug. Instead, here we looked at the ability of nicotine to amplify the reinforcing and incentive properties of a reward-predictive cue simply by being present during the acquisition and testing of the stimulus outcome relationship. Many studies demonstrating cue reactivity in humans are conducted in the absence of nicotine, avoiding any pharmacological effects on regional activation in the brain. Nicotine is capable of potentiating GABAergic transmission within the PFC, and so this pharmacological effect of the drug likely contributed to the reduction in cue-induced firing measured in our studies.

Although firing in the OFC was only directly measured in one experiment, the behavioral tests in Chapter 3 were designed to provide new understanding of the role of the OFC during Pavlovian approach behavior. We can interpret the behavioral results of Chapter 3 based on knowledge gained from other studies of the OFC as well as the circuit involved in Pavlovian approach behavior, although direct measurement of the OFC is required to confirm these hypotheses. We saw in Chapter 2 that the OFC was primarily phasically active during goal-tracking conditioned responses, and during receptacle entries when the rat was retrieving the reward. When the reward value was manipulated in Chapter 3, either by withholding the reward during extinction or by providing a different, less valuable reward by substituting water, rats were more likely to update their goal-tracking conditioned responding before the sign-tracking conditioned response. Based on previous lesion and inactivation studies of the OFC (Burke et

al., 2009; Chudasama and Robbins, 2003; Ostlund and Balleine, 2007; Panayi and Killcross, 2014) it is likely that the OFC was involved in updating the representation of the new stimulus-outcome association, and potentially in inhibiting the goal tracking conditioned response. Nicotine exposure further reduced the flexibility of conditioned responses when rats were presented with a change in the expected outcome. This suggests that the OFC was influenced by nicotine exposure, similar to other studies in which nicotine reduces behavioral flexibility on tasks that require new learning (Diergaarde et al., 2011; Hosking et al., 2014; Kolokotroni et al., 2014; Mendez et al., 2012). Reversal tasks require the OFC to integrate this new learning, suggesting that in the present experiments nicotine may have inhibited the ability of the OFC to update the behavior in response to the new outcome. The OFC was still functional, but was less likely to perform this action in animals exposed to nicotine compared to saline controls.

A limitation of these studies as a whole is that neuronal activity was only recorded from the OFC, and not from other regions of interest to this circuit. It is quite likely that the NAc, BLA, and VTA are all involved in both the expression of the Pavlovian conditioned approach behavior, and in updating the stimulus-outcome relationship during new learning. The BLA has been shown to be required for reversal tasks and updating the new value of a reward (Chang et al., 2012b; McGinty and Grace, 2008; Todd et al., 2014). In fact, neurons in the BLA may be faster to represent this new outcome than neurons in the OFC (Schoenbaum et al., 1998b), and the BLA and OFC are proposed to work synchronously to encode the value of stimuli and motivate behavior (Saddoris et al., 2005; Sharpe and Schoenbaum, 2016). To begin to test the involvement of both structures working together, we could perform electrophysiological recordings from both the BLA and OFC concurrently, and measure firing at different events during a Pavlovian task, similar to Chapter 2.

Nicotine is also capable of modulating neuronal activity in the NAc and VTA, and was most likely influencing both of these structures to produce the observed behavioral results

(Bassareo et al., 2007; Gotti et al., 2010; Liechti et al., 2007). It would be likely that during the behavioral challenges in Chapter 3 that manipulated the expected outcome, prediction error signaling by dopaminergic neurons in the VTA also played a role. We would expect that dopaminergic signaling from the VTA was involved in updating the value of the new outcome and the eventual adaptation of goal-tracking behavior to reflect this new stimulus-outcome association. Concurrent recording from neurons in both of these regions during the behavioral challenge sessions combined with a technique that could measure dopamine release such as fast scan cyclic voltammetry would provide additional information about the contributions of these interconnected regions during the expression of Pavlovian approach behaviors.

One general future direction to follow these studies is to evaluate the role of discrete populations of neurons in the OFC, based on their projections. For example, a recent study of dorsomedial prefrontal projections to the NAc and paraventricular nucleus of the thalamus (PVT) used *in vivo* two-photon calcium imaging to investigate neuronal firing in response to the presentation of a reward-predictive cue, and found that the firing pattern of dorsomedial PFC projection neurons was specific to the target region (Otis et al., 2017). While the projections in this particular study originated in a separate region of the PFC, both the NAc and PVT are proposed to be part of the circuit responsible for Pavlovian approach behaviors (Flagel and Robinson, 2017b). Measuring changes in neuronal activity based on specific projections from the OFC to target regions such as the striatum and BLA would contribute to our understanding of how this circuit influences behavior. These studies would be useful for identifying the components of Pavlovian approach behavior that are represented by connectivity between target regions, which would strengthen our knowledge gained from lesion or inactivation studies that describe contributions from regions when separated from the circuit itself.

In addition to measuring the activity of specific projections during the behavior, another informative experiment would be to directly manipulate these same projections to evaluate the

effect on conditioned approach. One recent study used optogenetics to directly manipulate the population of lOFC neurons that projects to the BLA, and the reciprocal projection from the BLA to lOFC. Inhibiting each pathway separately produced a dissociation in the function of the lOFC and BLA during cue-induced cocaine-seeking, finding that the projection from the lOFC to the BLA, but not the reciprocal projection, was required for this behavior (Arguello et al., 2016). It is possible that a similar study that inactivates the same monosynaptic projection between the lOFC and BLA may distinguish between the roles of the OFC and the BLA during a Pavlovian approach task, and the role of each region when updating behavior. An experiment to test this would be to manipulate the expected outcome using the tasks described in Chapter 3, and inhibit either pathway. Based on previous studies of this circuit, it is likely that the loss of either projection would reduce behavioral adaptation to a change in outcome value. I would hypothesize that inactivating the BLA to OFC pathway would reduce initial learning and prevent rapid updating of the behavior, while the inactivation of the OFC to BLA pathway would delay behavioral adaptation across multiple sessions. This same technique could be used to investigate the role of projections between other components of this circuit, such as the striatum and VTA. Directly activating or inhibiting projections from the OFC to the striatum or VTA could modify the expression of sign- or goal-tracking behaviors, as the current studies indicate that the OFC encodes goal tracking but not sign tracking. Inhibiting projections from the OFC to the ventral striatum may therefore reduce the expression of the goal tracking conditioned response, and increase the likelihood that an animal will sign track.

Additionally, other regions of the prefrontal cortex or subregions of the orbitofrontal cortex that were not investigated in this dissertation may be involved in the expression of Pavlovian approach. Both the anterior cingulate and medial prefrontal cortex have been proposed to be involved in the expression of Pavlovian approach (Campus et al., 2016; Homayoun and Moghaddam, 2009; Petykó et al., 2015; Tomie et al., 2004) and the mOFC may

provide a function separate from the lOFC (Gourley et al., 2016; Mar et al., 2011), which was the target of the current studies. Recording from both orbitofrontal regions, as well as additional regions of the PFC, would add a significant amount of knowledge to the understanding of Pavlovian conditioned approach and general knowledge of the individual contributions of separate regions of the PFC to control of behavior.

In Chapter 3 we conducted a dose-response curve using the opioid receptor antagonist naltrexone, and found that it reduced goal tracking specifically in NIC rats. This study was limited by low power due to a small number of animals in each group, specifically for a study of Pavlovian conditioned approach, which often requires large numbers of animals to measure both sign-and goal-tracking (Meyer et al., 2012). Nevertheless, the reduction of conditioned approach specific to goal tracking in nicotine-exposed rats is a promising result that warrants further exploration. A follow up study of this effect could utilize the challenge conditions that were also carried out in this chapter, combining naltrexone exposure with extinction or water substitution. As rats that were exposed to nicotine were resistant to extinction and extinguishing conditioned responses, it is possible that naltrexone would facilitate the change in behavior. The results of this experiment would translate to clinical studies that demonstrate a potential use for naltrexone as a smoking cessation aid. In clinical populations, the use of naltrexone for smoking cessation has yielded mixed results, as naltrexone has been shown to reduce cigarette craving and smoking in laboratory studies and after clinical treatment (Epstein and King, 2004; King et al., 2012; King and Meyer, 2000; Rohsenow et al., 2007; Rukstalis et al., 2005) but the effects and longevity of these outcomes vary between studies. Combination therapies using naltrexone can have a magnified effect when paired with nicotinic replacement therapy or other drugs used for nicotine cessation (Krishnan-Sarin et al., 2003; O'Malley et al., 2006). This suggests the importance of targeting multiple properties of nicotine for successful smoking cessation, as the drug has both primary rewarding and reinforcement-enhancing effects.

In Chapter 4, we expanded our investigation of nicotine-enhanced Pavlovian conditioned approach to include females, where the previous studies were completed in only males. The majority of studies that investigate the behavioral effects of nicotine or Pavlovian approach behavior have been completed using males, presumably because of concerns that arise with the use of females such as the influence of estrous cycle. One study of Pavlovian conditioning that did include females measured estrous cycle over time and correlated it with behavior, finding that it was not a significant source of individual variability in females (Pitchers et al., 2015). In our study, we found that females showed slightly more sign-tracking behaviors than males and were more likely to be classified as sign trackers. This is similar to a separate study from our lab (Madayag et al., 2017) in which adolescent rats were exposed to ethanol during adolescence and then trained on the Pavlovian conditioned approach procedure during adulthood. In that study, we also found that females were more likely to be sign trackers than males, a difference that was more pronounced than in the present study. Together, these data suggest that females are more likely to sign track than males, as this effect of sex emerged across different training cohorts and in animals that were ordered from a vendor or bred in-house.

To better interpret these differences between male and female rats, we assessed the variability if individuals in each sex or drug exposure group over 4 days of training to account for fluctuations that may occur due to estrous cycle. We found that females only showed more variability than males on the latency to enter the receptacle, indicating that estrous cycle does not contribute the variability responsible for the observed sex difference in sign tracking. While estrous cycle does not appear to contribute directly to Pavlovian approach, it is possible that circulating sex hormones could have organizational effects during development, or modulate behavior by influencing synaptic plasticity in adulthood. In this way, sex could be a significant source of individual variation in addiction vulnerability. Experiments conducted in humans report

sex differences on some behavioral tasks, and find variation in the effect of drugs of abuse. In some studies of human smokers, for example, female smokers show more reactivity to smoking-associated cues than males (Perkins et al., 1999). While we did not measure a sex difference in the ability of nicotine to enhance Pavlovian approach behaviors, it is possible that a sex difference could emerge under challenge conditions. If female rats are more sensitive to the incentive-amplifying effects of nicotine, as suggested by other studies (Chaudhri et al., 2006), then females in our Pavlovian approach paradigm may show sign tracking that is less flexible than males. We saw in Chapter 2 that both sign-and goal-tracking were reduced when nicotine was not injected immediately before the session in males. It could be that females would be less likely to reduce sign tracking even when nicotine is not pharmacologically active during the session.

The studies of expected outcome manipulation in Chapter 3 should also be replicated in female animals, as rats may show sex differences in behavioral inhibition. Females have been reported to exhibit increased impulsivity compared to males, as well as a decreased ability to inhibit behavior on tasks that measure impulsive action (Weafer and de Wit, 2014). A follow up study to the experiments in this dissertation would be to question if females are more likely to show slower extinction learning compared to males, and if nicotine exposure contributes to reduced behavioral flexibility in females. These experiments could further contribute to our understanding of sex-specific effects by investigating if the enhanced sign tracking seen in females is represented in the firing rate of neurons in regions related to these behaviors, such as the OFC. One potential experiment would be to investigate the baseline difference between males and females in response to a reward-predictive cue, with or without drug exposure. There may also be differences in sensitivity to naltrexone and opioids that could be uncovered using this Pavlovian conditioned approach paradigm. We found that naltrexone specifically reduced goal tracking in males exposed to nicotine, and evaluating the effect of the opioid antagonist in

females that may have different opioid sensitivity could contribute to our understanding of the effects of naltrexone in clinical populations (Covey et al., 1999; Epstein and King, 2004; Ray et al., 2006).

In the final experiment of Chapter 4, we measured expression of BDNF protein in the OFC, NAc, and BLA. We chose to measure BDNF because it was at an interesting intersection where protein expression is influenced by nicotine administration (Ortega et al., 2013; Parikh et al., 2016), differentially expressed in sign- and goal-tracking animals (Morrow et al., 2015), expressed in the OFC and other target areas (Conner et al., 1997; Pitts et al., 2016) and potentially involved behavior (Gourley et al., 2016; Zimmermann et al., 2015). However, BDNF levels in the various brain regions were not simply correlated with conditioned responses. Instead, we found that the ratio of BDNF protein expression in the OFC and NAc was correlated with latency to press the lever only in NIC females. It does seem that plasticity may be altered in nicotine-exposed animals, resulting in an interaction between sex and protein expression that contributes to behavior, but future studies will need to confirm this possibility.

Corticostriatal BDNF has been shown to differentially regulate cocaine-seeking, as BDNF infusion into limbic or striatal regions potentiates while prefrontal BDNF expression blunts cocaine-seeking (Ghitza et al., 2010; Pitts et al., 2016). A similar relationship between corticostriatal BDNF is predicted for alcohol-seeking as well (Logrip et al., 2015; Warnault et al., 2016). It is possible that this relationship exists for nicotine, but it has not been confirmed. OFC and striatal BDNF are differently required for habitual behaviors, as depletion of BDNF in the mOFC but not the striatum reduced behavioral flexibility, and behavior was recovered by infusion of BDNF protein directly into the mOFC (Gourley et al., 2016). Although the majority of this research points to habitual actions driven by the dorsal striatum, projections from the OFC to the ventral striatum and the potential secretion of BDNF into the ventral striatum may contribute specifically to Pavlovian approach behaviors. It would be interesting to investigate if

direct manipulation of BDNF in the OFC could specifically blunt the sign-tracking conditioned response or make it more flexible after a change in the expected outcome. Further study of the ability of prefrontal and striatal BDNF to directly influence behavior would continue to pull apart the influence of corticostriatal circuitry on behavior.

Future studies could continue to specifically investigate this predicted difference in protein expression and how it relates to behavior using Pavlovian conditioned approach. As direct infusion of BDNF into the mOFC can recover behavioral flexibility (Gourley et al., 2016), one interesting study would be to infuse BDNF into the OFC of animals that show sign tracking behavior, and then challenge them to adapt their behavior during an extinction task. BDNF in both the mOFC and lOFC have been shown to be directly involved in goal-directed behavior (Gourley et al., 2016; Zimmermann et al., 2015), and so precise targeting of these structures could further dissociate the influence of OFC subregions on Pavlovian approach. BDNF expression was not significantly modified across experimental conditions in the studies for this dissertation, but it is possible that we did not detect a difference in BDNF because we measured protein expression at a time where there was no new learning or need for synaptic plasticity. Using the challenge conditions from Chapter 3 could produce an opportunity to test the hypothesis that BDNF protein in the OFC will be reduced in sign tracking animals, or in animals that show less flexible responding after nicotine exposure.

The significance of the OFC/NAc ratio for BDNF protein expression also speaks to a potential general imbalance of activation and control of behavior between the PFC and striatum, seen in animals across multiple drugs of abuse and behavioral traits (Everitt and Robbins, 2016). Addiction has been described as a shift from goal directed to compulsive or habitual behaviors, and this shift is proposed to directly involve a transition from top down or inhibitory control by the PFC, to enhanced reward-seeking or compulsive actions controlled by the striatum (Everitt et al., 2008). Pavlovian conditioned approach could be used to investigate this

transition, to establish if a shift in behavior occurs similar to that seen with goal directed and habitual or compulsive behaviors. While sign tracking is not the same as habitual responding, both behaviors share overlapping neuronal circuitry and the hallmark characteristic of inflexibility. An interesting future direction will be to directly investigate the neuronal circuity that promotes sign tracking during Pavlovian approach behavior and the development of habitual instrumental responding.

Conclusions

Taken together, the results of this dissertation demonstrate encoding of Pavlovian conditioned approach responses in the OFC, and the relative strength of these conditioned responses after nicotine exposure. We demonstrate that nicotine reduces the flexibility of these behaviors and identify the need to continue to investigate sex differences in behaviors that confer addiction vulnerability. Additionally, we show that pharmacological manipulations can be used to target nicotine-enhanced conditioned responding, and we identify a possible protein of interest with translational relevance to smoking and addiction-associated behaviors. The results of these studies confer a translational impact, as this animal model of Pavlovian conditioned approach can be used to investigate the attribution of salience to nicotine-associated stimuli in populations of human smokers.

Nicotine enhancement of the salience of conditioned stimuli is a significant problem in clinical populations of smokers, as exposure to these drug-associated stimuli can provoke the experience of craving. Attempting to disrupt these conditioned associations or to block the amplification of salience of a nicotine-associated cue is a monumental but important task, as current treatments such as nicotine replacement or pharmaceutical therapies alone do not appear to significantly reduce cue-induced craving. Here, we have shown that the Pavlovian conditioning paradigm can be used to measure this enhanced attribution of salience of cues and to manipulate this property of nicotine. One avenue of future studies with translational impact

could use Pavlovian conditioned approach behavior to test pharmacological interventions that target the incentive salience of the conditioned cue and then block the sign-tracking approach response specifically. Continued investigation of the ability of conditioned cues to motivate behavior, and the underlying neurobiological substrates that contribute to this effect, will possibly lead to new and more successful treatment options to benefit smokers as well as those affected by multiple substance abuse disorders.

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