EXAMINATION OF TOLERANCE TO THE COGNITIVE ENHANCING EFFECT OF NICOTINE ON CONTEXTUAL CONDITIONING

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By
Derek S. Wilkinson
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Examination Committee:

Thomas Gould, Psychology
Kim Curby, Psychology
Jason Chein, Psychology
Robert Weisberg, Psychology
Tania Giovannetti, Psychology
Vinay Parikh, Psychology

ABSTRACT

Nicotine addiction is a multifaceted disease that can be influenced by several factors. Emerging evidence indicates that the neural substrates of nicotine addiction overlap with the neural substrates of learning and memory. Nicotine modulates various types of learning and memory and the ability of nicotine to alter cognitive processes may contribute to its addictive liability. Acute nicotine enhances contextual conditioning in mice, tolerance develops to this effect with chronic administration, and withdrawal from chronic nicotine produces cognitive deficits. While tolerance and withdrawal deficits both occur following chronic administration, it is unknown if they share similar mechanisms. The series of experiments in Chapter 2 were designed to provide evidence that tolerance and withdrawal are dissociable. C57BL/6J mice were implanted with osmotic minipumps that delivered constant nicotine or saline for various durations and then were trained and tested in contextual conditioning either during chronic nicotine administration or 24 hours after pump removal. Chronic nicotine enhanced contextual conditioning in a dose- and time-dependent manner. Tolerance developed quickly to the enhancing effect of chronic nicotine. Furthermore, the duration of chronic nicotine treatment required to produce cognitive deficits upon cessation of treatment differed than that required to produce tolerance, which suggests that tolerance and withdrawal are mediated by separate mechanisms. Chapter 2 concludes by presenting a model that integrates nicotinic acetylcholine receptor desensitization and upregulation to explain the present findings.

The model presented in Chapter 2 predicts that there will be enhanced sensitivity to acute nicotine during a period of nicotine withdrawal. Previous research indicates that prior exposure to nicotine enhances sensitivity to acute nicotine injections, but it is

unclear if this enhanced sensitivity is due to prior nicotine exposure or enhanced sensitivity to nicotine during withdrawal. Therefore, the experiments in Chapter 3 were designed to determine if prior exposure to nicotine or nicotine withdrawal altered sensitivity to acute nicotine injections. This was accomplished by assessing the effects of acute nicotine on contextual conditioning immediately after cessation of chronic nicotine treatment and two weeks later, a time period not associated with withdrawal-related changes in cognitive function. Results of the study showed that acute nicotine enhanced contextual conditioning across a wide range of doses in both saline- and nicotine-withdrawn mice. However, a greater enhancement of contextual conditioning was observed in mice withdrawn from chronic nicotine treatment for 24 hours than all other withdrawal groups, suggesting enhanced sensitivity during withdrawal. The enhanced sensitivity to acute nicotine suggests altered nAChR function during withdrawal. In addition, the lowest dose of acute nicotine did not enhance contextual conditioning in groups that received chronic nicotine but did in other groups. The simultaneous observation of a hyper and hyposensitive nAChR system during withdrawal suggests that there may be a phasic response to chronic nicotine. Together, the results of the present study suggest that tolerance and withdrawal operate under separate mechanisms, and that there is overall enhanced sensitivity to nicotine during periods of nicotine withdrawal.

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TABLE OF CONTENTS

Page

LIST OF FIGURES

Figure Page

LIST OF TABLES

CHAPTER 1

INTRODUCTION

Tobacco products are some of the most widely used drugs and are the leading preventable cause of death. It is estimated that more than 5 million people die per year from tobacco products (WHO, 2009). In the United States, cigarette smoking results in an estimated 443,000 premature deaths each year and costs $193 billion in direct health care costs and productivity losses each year (CDC, 2011). The Substance Abuse and Mental Health Services Administration's National Survey on Drug Use and Health reported that in 2010, an estimated 69.6 million Americans aged 12 or older were current users of a tobacco product, which represents approximately 27.4% of the population (SAMHSA, 2010). Nicotine in tobacco is highly addictive resulting in relatively low successful quit rates. The addictive properties of nicotine are illustrated by the fact that over 70% of smokers wish to quit, 40% make an attempt every year, and yet only 3 to 5% of these smokers are successful at quitting (Nides, 2008). Nicotine has relatively weak reinforcing properties (Palmatier et al., 2006), suggesting factors other than reinforcement are responsible for the highly addictive nature of nicotine. Emerging evidence indicates that the neural substrates of nicotine addiction overlap with the neural substrates of learning and memory (Gould, 2006; Hyman, 2005; Kelley, 2004). Thus, one potential contributor to nicotine addiction may be the effects of nicotine on learning and memory (Gould, 2006).

There are at least two ways in which the ability of nicotine to alter learning and memory may contribute to its addictive liability. First, the acute effects of nicotine, which enhance learning and memory, might contribute to nicotine addiction. Acute nicotine enhances learning and memory including spatial and contextual learning (Davis,

Porter, & Gould, 2006; Gould & Higgins, 2003; Gould & Wehner, 1999; Kenney, Adoff, Wilkinson, & Gould, 2011; Kenney & Gould, 2008b). Nicotine might usurp the neural substrates of normal learning and memory to gain powerful control over behavior. Specifically, the cognitive enhancing effects of acute nicotine may aid in the formation of maladaptive drug-context associations, which can elicit cravings and trigger relapse. Second, nicotine withdrawal-deficits in cognition can also contribute to nicotine addiction by maintaining nicotine use. Withdrawal from chronic nicotine produces a number of withdrawal symptoms including cognitive deficits (Davis, James, Siegel, & Gould, 2005; Jacobsen et al., 2005; Jacobsen, Mencl, Constable, Westerveld, & Pugh, 2007; Patterson et al., 2010). These cognitive deficits can be avoided or alleviated with nicotine, which may aid in the maintenance of nicotine use. This is important, as changes in cognition during nicotine withdrawal can predict relapse (Patterson, et al., 2010).

Fear conditioning has been a fruitful paradigm for examining the effects of nicotine on learning and memory (Kenney & Gould, 2008a). In a typical fear conditioning procedure, animals are placed into training chambers, and after a period of time, are presented with an auditory conditional stimulus (CS) that co-terminates with an aversive footshock unconditional stimulus (US). As a result of this training, two different associations are formed: one between the CS and the US (cued conditioning) and another between the context and the US (contextual conditioning). Typically, one to two CS-US pairings during training is sufficient to produce robust learning. The next day, animals are placed in the original training context in the absence of the CS to assess contextual conditioning. Afterward, the animals are placed in an altered context in the presence of the CS to assess cued conditioning. The association between the CS and the US does not

require the hippocampus while the association between the context and the US requires does (Logue, Paylor, & Wehner, 1997; Phillips & LeDoux, 1992). Therefore, one training session allows for subsequent analysis of both hippocampus-dependent and hippocampus-independent forms of learning and memory.

Acute systemic administration of nicotine prior to training and testing of fear conditioning enhances contextual conditioning in a dose-dependent manner (Gould & Higgins, 2003; Gould & Wehner, 1999). Infusions of nicotine directly into the hippocampus also dose-dependently enhance contextual conditioning, confirming hippocampal involvement in the acute enhancing effect of nicotine (Davis, Kenney, & Gould, 2007). Chronic systemic administration of nicotine, at a dose that produces the same plasma nicotine levels as an acute enhancing dose and chronic hippocampal infusion of nicotine both produce no change in contextual conditioning (Davis & Gould, 2009; Davis, et al., 2005). On the other hand, withdrawal from this same dose of chronic nicotine and withdrawal from chronic hippocampal infusion of nicotine both impair contextual conditioning (André, Gulick, Portugal, & Gould, 2008; Davis & Gould, 2007; Davis & Gould, 2009; Davis, et al., 2005; Portugal & Gould, 2009; Raybuck, Portugal, Lerman, & Gould, 2008; Wilkinson & Gould, 2011). None of these studies report a significant effect on cued conditioning. Thus, these studies indicate that nicotine specifically interacts with the neural substrates of hippocampus-dependent forms of learning and memory rather than cognitive processes involved in multiple other types of learning and memory, such as attention or arousal. Taken together, these results indicate that nicotine acts within the hippocampus to produce differential effects on contextual

conditioning depending upon the pattern of administration (i.e., acute, chronic, or withdrawal from chronic nicotine).

The development of tolerance to the enhancing effect of nicotine with chronic administration and the appearance of withdrawal-deficits in contextual conditioning upon cessation of treatment suggest that chronic nicotine alters hippocampal processes involved in contextual conditioning. However, it is unknown if the same processes involved in tolerance are also involved in withdrawal-deficits. As both occur following chronic administration, theories of tolerance and withdrawal posit that they are inextricably linked (Benowitz, 2010; File, Baldwin, & Aranko, 1987; Leventhal & Cleary, 1980; Poulos & Cappell, 1991). Namely, tolerance is a homeostatic response to compensate for drug-induced changes in physiological function, and withdrawal is a manifestation of that homeostatic response in the absence of the drug. In this view, both tolerance and withdrawal reflect the same homeostatic adaptation; they are two outputs of the same process (Poulos & Cappell, 1991). However, emerging evidence indicates that tolerance and withdrawal are separate phenomena (DiFranza & Wellman, 2005; Perkins, 2002; Perkins et al., 2001). For example, there is a subset of smokers who do not meet the criteria for tobacco dependence and do not experience withdrawal effects following cessation of tobacco use (termed nondependent smokers or "chippers"), but who do show tolerance to nicotine (Perkins, 2002; Perkins, et al., 2001). Tolerance to many of the subjective effects of nicotine is similar between dependent (smoked at least ten cigarettes per day for the past ten years) and nondependent (six or fewer cigarettes per day but smoked on at least five days per week with no past history of regularly smoking) smokers (Perkins, et al., 2001), which indicates that tolerance can occur in the absence of

withdrawal. Furthermore, abstinent smokers (~ seven years) also show tolerance to the subjective effects of nicotine (Perkins, et al., 2001) indicating that tolerance is long-lasting, outliving any withdrawal symptoms. In addition, recent evidence demonstrates that strains of mice that show nicotine withdrawal-deficits in contextual conditioning do not always show acute nicotine enhancement of contextual conditioning and vice versa (Portugal, Wilkinson, Kenney, Sullivan, & Gould, 2012). For example, C3H/HeJ mice do not exhibit acute nicotine enhancement of contextual conditioning. In addition, 12 mg/kg/d chronic nicotine for 13 days does not alter contextual conditioning. However, 24 hours of withdrawal from 12 days of 6.3 and 12 mg/kg/d chronic nicotine impairs contextual conditioning. If withdrawal is a manifestation of the same processes involved in tolerance (e.g., acute administration leads to a homeostatic response that pulls the system back to baseline levels), then it would be expected that this strain of mice would not exhibit withdrawal-related deficits in contextual conditioning, as it did not exhibit acute nicotine enhancement or any alterations in contextual conditioning with chronic administration.

There have been relatively few animal studies that have directly examined the relationship between tolerance and withdrawal. Therefore, the goal of the present thesis is to examine that relationship. The experiments in Chapter 2 were designed to determine if tolerance and withdrawal operate under the same mechanisms. If tolerance and dependence share similar mechanisms, then the conditions that are sufficient to produce tolerance should also produce withdrawal. Comparing when tolerance first develops to chronic nicotine, and the duration of treatment required to produce withdrawal effects, will suggest if they share similar or separate mechanisms. Demonstration that tolerance

and withdrawal occur at the same time will provide evidence that they share the same mechanisms. However, demonstration that they occur at different times will provide evidence that they are indeed dissociable. Prevailing theories about the underlying mechanisms involved in withdrawal-related changes in behavior suggest that the nAChR system is hyperexcitable during withdrawal (Dani & Heinemann, 1996; Gould et al., 2012). This theory implies that there would be enhanced sensitivity to acute nicotine during a period of nicotine withdrawal. The series of experiments in Chapter 3 was designed to test the hypothesis that the nAChR system is hypersensitive during nicotine withdrawal. Mice were withdrawn from chronic nicotine treatment and either after 24 hours or 14 days were trained and tested in contextual conditioning following injections of acute nicotine. A greater enhancement of contextual conditioning with acute nicotine during a 24 hour nicotine withdrawal period relative to animals withdrawn from chronic saline would suggest a hypersensitive nAChR system. Examining the effect of acute nicotine on contextual conditioning during (24 hours) and after withdrawal (14 days) offers a comparison between the effects of prior nicotine exposure vs. nicotine withdrawal on contextual conditioning to determine if there is indeed a hypersensitive nAChR system during withdrawal.

CHAPTER 2

THE EFFECTS OF CHRONIC NICOTINE AND WITHDRAWAL FROM CHRONIC NICOTINE ON CONTEXTUAL CONDITIONING

Introduction

Chronic exposure to nicotine can result in at least two adaptations: dependence and tolerance (Benowitz, 2008, 2010). Nicotine dependence is a state induced by chronic exposure to nicotine that results in the appearance of a withdrawal syndrome upon cessation of nicotine treatment. Laboratory studies of nicotine dependence have shown that withdrawal can produce a number of somatic signs (Damaj, Kao, & Martin, 2003; Malin et al., 1992), decrease brain reward function (Epping-Jordan, Watkins, Koob, & Markou, 1998), increase anxiety (Damaj, et al., 2003), disrupt sustained attention (Shoaib & Bizarro, 2005), and produce deficits in learning and memory (Davis, et al., 2005). Tolerance, on the other hand, is an adaptation that results in nicotine losing its efficacy at producing an expected response and in higher doses of nicotine being required to produce that expected response (Goforth, Murtaugh, & Fernandez, 2010; Kalant & Khanna, 1990). Laboratory studies of tolerance have shown that chronic nicotine decreases sensitivity to many of the behavioral and physiological effects of nicotine (Damaj, 2005; Grabus et al., 2005; Marks, Burch, & Collins, 1983; Marks, Campbell, Romm, & Collins, 1991; Marks & Collins, 1985; Marks, Romm, Gaffney, & Collins, 1986; Marks, Stitzel, & Collins, 1985). With regard to cognitive enhancement, tolerance and dependence are both important factors in maintaining nicotine use. The logic is as follows: a person who has developed tolerance to the cognitive-enhancing effects of nicotine will need to self-administer more nicotine than previously to compensate. More smoking would then lead

to greater dependence (Perkins, 2002) and greater withdrawal-associated cognitive deficits, which can be avoided or alleviated by continued smoking. This is important, as changes in cognition during withdrawal contribute to and can predict relapse (Patterson, et al., 2010). Although both tolerance and withdrawal occur following chronic nicotine administration, the relationship between tolerance and withdrawal is unclear and it is unknown if they share similar mechanisms.

The effects of chronic and withdrawal from chronic nicotine on various types of hippocampus-dependent learning and memory has been the focus of much research. However, the relationship between nicotine tolerance and withdrawal in these models of cognitive function remains unclear. One such model is the radial arm maze. The radial arm maze is a hippocampus-dependent task that may be used to study spatial working memory (Meck, Church, & Olton, 1984). In the radial arm maze, chronic nicotine enhances working memory, with no indication of tolerance or withdrawal effects upon cessation of treatment (Levin, Briggs, Christopher, & Rose, 1992; Levin, Christopher, Briggs, & Rose, 1993; Levin et al., 1990). Even during a period of withdrawal after chronic exposure, animals that receive nicotine still perform better than saline-treated controls (Levin, et al., 1990). The facilitated performance during nicotine withdrawal was not due to nicotine administration during training, as rats trained during nicotine withdrawal had better performance relative to controls (Levin, Briggs, Christopher, & Rose, 1992). Thus, evidence suggests that, at least in the radial arm maze, chronic nicotine can improve working memory with no indication of tolerance developing or withdrawal-related cognitive impairments.

Much like in the radial arm maze, chronic nicotine enhances spatial learning in the Morris water maze with no evidence of tolerance or withdrawal (See Table 1). The Morris water maze is a frequently used paradigm to assess spatial learning (Morris, Garrud, Rawlins, & O'Keefe, 1982). The hippocampus is a crucial neural substrate for spatial learning and therefore the Morris water maze is considered a hippocampus-dependent task (Logue, et al., 1997). In the Morris water maze, rats given 10 days of chronic nicotine treatment show enhanced spatial learning when tested 24 hours after the last drug injection (Abdulla et al., 1996), showing no nicotine-withdrawal impairments in learning. In another study, mice given 5 days of chronic nicotine treatment prior to training and 4 days of chronic nicotine treatment through training had better maze performance than saline treated controls, with no evidence of tolerance (Bernal, Vicens, Carrasco, & Redolat, 1999).

A lack of tolerance was also found in another study, where rats in two different conditions each received twice-daily injections of 0.35 mg/kg of nicotine spaced 5 h apart for 14 days (Hernandez & Terry, 2005). Rats in the daily single-trial condition were trained between each injection while rats in the single-day, multiple trial conditioned received one day of extensive training 21 h following the final injection. Results of the study showed there were no significant differences between nicotine-treated and control rats in locating the submerged platform in the single-day, multiple-trial group. However, nicotine-treated rats in the daily single-trial condition were more efficient than control rats in finding the submerged platform, indicating enhanced spatial learning with no tolerance or withdrawal (Hernandez & Terry, 2005). Thus, nicotine enhances spatial learning in the Morris water maze with no indication of tolerance or withdrawal.

Table 1. Summary of Morris water maze findings sc = subcutaneous; ip = intraperitoneal; mp = minipump

Author	Year	Subjects	Dose	ROA	Treatment Before Task	Treatment During Task	Result
Abdulla	1996	Male Sprague Dawley Rats	0.7 mg/kg	sc	10 days, 2 doses/day	-	Enhancement
Bernal	1999	Male C57BL/6J Mice	0.35 and 0.7 mg/kg	sc	5 days, 1 dose/day	15 min prior to 4 days of training	Enhancement
Hernandez	2005	Male Wistar Rats	0.35 mg/kg	sc	-	5 hours before and after 14 days of training	Enhancement
Socci	1995	Male Sprague-Dawley (2-3 mon)	0.07 mg/kg	ip	3 days	15 min prior to 7 days of training	Enhancement
		Male Sprague-Dawley (25-26 mon)	0.07 mg/kg	ip	4 days	15 min prior to 9 days of training	No effect
Attaway	1999	Fischer 344 Rats (4 mon)	0.2 mg/kg	ip	-	15 min prior to training	No effect
		Fischer 344 rats (24 mon)	0.2 mg/kg	ip	-	15 min prior to training	No effect
Moragrega	2003	Male NMRI Mice (group housed)	0.35 and 0.175 mg/kg	sc	-	15 min prior to 5 days of training	No effect
		Male NMRI Mice (individually housed)	0.35 and 0.175 mg/kg	sc	-	15 min prior to 5 days of training	Impairment
Scerri	2006	Male Sprague Dawley Rats	0.25 and 4 mg/kg	mp	2 days	4 days	Impairment with 4/mg/kg
		Male Sprague Dawley Rats	0.25 and 4 mg/kg	mp	2 days	4 days	Nonsignificant enhancement with 0.25 mg/kg

While some studies found a nicotine-induced enhancement of spatial learning (Abdulla, et al., 1996; Bernal, et al., 1999; Hernandez & Terry, 2005), other studies have found no effect of nicotine or a nicotine-induced impairment of spatial learning (Attaway, Compton, & Turner, 1999; Moragrega, 2003; Scerri, Stewart, Breen, & Balfour, 2006; Socci, Sanberg, & Arendash, 1995). This inconsistency in findings is most likely a result of a number of factors, including the dose of nicotine, the length of training time, and the strain/age of the animals tested (See Table 1). For example, Scerri et al (2006) found that infusion of nicotine through osmotic minipumps at a dose of 4 mg/kg/d produced a modest impairment in acquisition of the Morris water maze and significantly impaired retention of spatial memory when tested with a probe trial. On the other hand, rats that received 0.25 mg/kg/d nicotine through the same route of administration showed enhanced acquisition of the task, although not to statistically significant levels (Scerri, et al., 2006). Thus, the doses of nicotine used in Morris water maze studies have a significant impact on task performance. Another study examining nicotine's effects on Morris water maze performance found that nicotine did not improve performance in either individually or group-housed NMRI mice (Moragrega, 2003). In fact, both doses of nicotine (0.35 and 0.175 mg/kg) administered once daily prior to training sessions (four sessions per day for 5 days) actually impaired acquisition in individually housed mice (Moragrega, 2003).

The age of the animals tested also has a significant influence on nicotine's effects on spatial learning. In one study, daily pre-treatment with 0.07 mg/kg nicotine for 3 days prior to initial training led to an increase in spatial learning in aged but not young rats (Socci, et al., 1995). However, young rats only received 7 days of training while aged

rats received 14 days of training (Socci, et al., 1995). However, another study demonstrated that administration of 0.2 mg/kg nicotine prior to training had no effect on performance in either age group (Attaway, et al., 1999). Clearly, multiple factors influence the effects of nicotine on Morris water maze performance, such as the dose of nicotine, housing conditions, the age of the animals, and the duration of training time. It should be noted that several studies utilized a once daily injection schedule, which makes it difficult to determine if acute or chronic nicotine was examined.

Chronic nicotine has also been shown to have an impairing effect on other hippocampus-dependent tasks. Object recognition examines a rodent's natural propensity to explore novel or spatially displace objects over familiar or spatially static objects. Acute, chronic, and withdrawal from chronic nicotine produce differential results on novel and spatial object recognition. Chronic nicotine and withdrawal from chronic nicotine produce impairments in spatial object recognition, which is believed to be a hippocampus-dependent task, while acute nicotine enhances spatial object recognition (Kenney, et al., 2011). The withdrawal-deficit in spatial objection recognition is unlikely due to the same processes involved in the deficit due to chronic nicotine administration, because mice treated with chronic nicotine demonstrated recognition of the spatially displaced object while mice undergoing nicotine withdrawal did not. On the other hand, acute nicotine impairs novel object recognition, which is believed to be a hippocampus-independent task. Tolerance develops to this effect with chronic administration, and withdrawal has no effect (Kenney, et al., 2011). The differential effects of acute, chronic, and withdrawal from chronic nicotine on spatial and novel object recognition suggest

differing underlying neural substrates involved in these tasks. These results also suggest that tolerance can develop to the impairing effects of nicotine.

On the other hand, some studies report that both tolerance and withdrawal-deficits in hippocampus-dependent learning occur with chronic nicotine administration. While acute nicotine enhances contextual conditioning (Gould & Higgins, 2003), chronic nicotine has no effect, suggesting tolerance with chronic exposure (Davis, James, Siegel, & Gould, 2005). In addition, withdrawal from chronic nicotine impairs contextual conditioning (Davis et al., 2005). This deficit in contextual conditioning was found to be due to impaired learning rather than impaired recall (Portugal & Gould, 2009). Importantly, the dose of chronic nicotine used in the tolerance and withdrawal study produced the same plasma nicotine levels as an acute enhancing dose (Davis, et al., 2005). Unlike the radial arm maze, Morris water maze, and spatial object recognition, tolerance and withdrawal effects in contextual conditioning are both evident following chronic administration. Therefore, contextual conditioning is an excellent model of cognitive function to dissociate tolerance from withdrawal.

The differential effects of chronic and withdrawal from chronic nicotine across tasks that involve the hippocampus are likely due to several factors. As with the Morris water maze, the age of the animals, dose of nicotine used, and duration of training influences the effects of nicotine on learning and memory (Scerri, et al., 2006; Socci, et al., 1995). In addition, different neural substrates and cognitive processes mediate each of the learning and memory paradigms discussed above, and nicotine likely produces differential effects depending on which of these substrates are sensitive to nicotine (Hodges, 1996; Kenney, et al., 2011; Kenney & Gould, 2008; Postma, Kessels, & van

Asselen, 2008). For example, object recognition is an incidental learning task, the radial arm maze is an appetitively motivated task, while the Morris water maze and fear conditioning are aversively motivated tasks (Hodges, 1996; Phillips & LeDoux, 1992; Postma, et al., 2008). Task motivation is likely to not only affect learning and memory strategies or processes (e.g., maze performance relies on search strategies while fear conditioning is an associative learning paradigm) but also nicotine's effects on each task. In addition, nicotine differentially affects hippocampal subregions, which influences task performance. Nicotine infused into the dorsal hippocampus enhances contextual conditioning while nicotine infused into the ventral hippocampus impairs contextual conditioning (Davis, et al., 2007; Kenney, Raybuck, & Gould, 2012; Raybuck & Gould, 2010). Antagonism of high-affinity, but not low-affinity, nAChRs in the dorsal hippocampus blocks the enhancing effect of acute systemic nicotine on contextual conditioning (Davis, et al., 2007), while antagonism of both high-affinity and low-affinity nAChRs in the ventral hippocampus does not block the enhancing effect of acute systemic nicotine on contextual conditioning (Kenney, et al., 2012). On the other hand, antagonism of low-affinity, but not high-affinity, nAChRs in the ventral hippocampus blocks the enhancing effect of nicotine in the radial arm maze (Bancroft & Levin, 2000; Bettany & Levin, 2001). It is unknown how antagonism of high- and low-affinity nAChRs in the dorsal hippocampus alters the effects of nicotine on radial arm maze performance. Thus, multiple factors can contribute to the differential effects of nicotine across learning and memory paradigms.

Along with the behavioral observations of tolerance and withdrawal, chronic nicotine also produces two cellular adaptations that may underlie both tolerance and

withdrawal. Chronic nicotine desensitizes nAChRs (Gentry & Lukas, 2002; Marks, Grady, & Collins, 1993; Marks, Grady, Yang, Lippiello, & Collins, 1994; Ochoa, Chattopadhyay, & McNamee, 1989; Ochoa, Li, & McNamee, 1992). As receptor desensitization is a reduction in receptor function, it is believed that desensitization is one mechanism that underlies tolerance (Robinson et al., 2007). The second cellular adaptation in response to chronic nicotine is nAChR upregulation, an increase in binding sites (Marks, et al., 1983; Marks, et al., 1985). Nicotine is paradoxical in that, unlike other drugs of abuse, nicotine upregulates rather than downregulates its own receptors (Wonnacott, 1990). Upregulation has been suggested to be a homeostatic response to receptor desensitization and was originally thought to underlie tolerance (Marks, et al., 1983; Marks, et al., 1985), but there is not always a parallel between receptor upregulation and tolerance (McCallum et al., 2000). Recent data from our laboratory, however, suggest that upregulation of high-affinity nAChRs in the hippocampus mediates withdrawal-related deficits in contextual conditioning (Gould, et al., 2012). Namely, the return of upregulated receptors to control levels matches the time course of nicotine withdrawal-deficits in contextual conditioning. In addition, we have found that strains and ages of mice that do not show nicotine withdrawal-deficits in contextual conditioning also do not show upregulation of high-affinity nAChRs in the dorsal hippocampus (Portugal, Wilkinson, Turner, Blendy, & Gould, in press). Thus, the data to this point appear to implicate nAChR desensitization to tolerance (Robinson, et al., 2007) and upregulation to withdrawal (Gould, et al., 2012).

The aim of the present study was to test the hypothesis that tolerance to the enhancing effect of nicotine and nicotine withdrawal-deficits in contextual conditioning

are dissociable. Contextual conditioning is a good model for dissociating tolerance and withdrawal, as both occur following the same dose. It is hypothesized that a threshold of chronic nicotine treatment must first be reached in order to produce significant changes that result in tolerance and a different threshold of chronic treatment will be required to produce withdrawal-deficits upon cessation of treatment. Determining if the conditions that are sufficient to produce tolerance also produce withdrawal would suggest similar underlying mechanisms. However, tolerance and withdrawal appearing at separate times would suggest that they operate under different mechanisms.

Method

Subjects

Subjects were male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) aged 8-12 weeks at the beginning of pump implantation. Mice were housed 1-4 per cage with *ad libitum* access to food and water. A 12-hour light/dark cycle was maintained from 7:00 AM to 7:00 PM with all experiments conducted during the light cycle. The Temple University Institutional Animal Care and Use Committee approve all experimental procedures.

Surgery

Mice were implanted with subcutaneous osmotic minipumps (Alzet, Model 1002, Durect Co, Cupertino, CA) that delivered chronic saline or nicotine at a rate of 0.25 µl/hr for 1 – 6 days depending on the dose of nicotine. Osmotic minipumps were surgically inserted subcutaneously via an incision in the lower back of the mouse. Surgery was

performed under sterile conditions with 5% isoflurane as the anesthetic. A second similar surgery was performed to remove pumps and induce nicotine withdrawal 1-4 days after pump implantation for withdrawal studies.

Drugs and Duration of Treatment

Nicotine hydrogen tartrate salt (Sigma, St. Louis, MO) was dissolved in 0.9% saline. Osmotic minipumps were filled with a 100 µl of solution that contained saline, or 3, 6.3, 12, or 24 mg/kg/d nicotine. Only 6.3 mg/kg/d nicotine or saline was used for nicotine withdrawal studies. For nicotine withdrawal studies, pumps were removed 1-4 days after implantation. All doses are reported as the freebase weight of nicotine.

Experiments and Conditions

To determine when tolerance develops to the enhancing effect of chronic nicotine at different doses, separate groups of mice were implanted with osmotic minipumps that delivered chronic saline or nicotine (3, 6.3, 12, or 24 mg/kg/d) for 1-6 days, depending on the dose, and were then trained and tested in contextual conditioning. Each dose was treated as a separate experiment and had a corresponding saline control resulting in four separate experiments with two groups per experiment (nicotine and saline). One dose of nicotine, 24 mg/kg/d, was found to produce deficits in contextual conditioning after one day of treatment. Therefore, a fifth experiment was performed in a separate group of mice, to determine if this deficit was specific to contextual conditioning or due to a global deficit in learning and memory, by testing the animals in delay fear conditioning, which has a hippocampus-dependent and -independent component.

To determine how many days of chronic nicotine are required to produce deficits in contextual conditioning upon withdrawal, separate groups of mice were implanted with osmotic minipumps that delivered saline or nicotine (6.3 mg/kg/d) for 1-4 days. Each duration of treatment had a chronic nicotine and saline component resulting in eight total groups.

Apparatus

Mice were trained and tested for contextual conditioning in four identical clear Plexiglas chambers (26.5 x 20.4 x 20.8 cm) housed in sound attenuating boxes (Med-Associates, St. Albans, VT). The floor of each chamber was made of metal bars (0.20 cm diameter) spaced 1.0 cm apart and connected to a shock generator and scrambler (Med Associates, Model ENV-414). Ventilation fans were mounted on the sides of each box to provide background noise. A 4 W light mounted above each box provided illumination. Stimulus administration was controlled by a PC running LabView software. Testing for cued conditioning occurred in an altered context consisting of four chambers (20.3 x 22.9 x 17.8 cm) housed in sound attenuating boxes (Med-Associates, St. Albans, VT) in a room different from the room in which animals were trained. The floor of each chamber was made of white, opaque plastic. Speakers were mounted on the left wall of each chamber that delivered the auditory CS. Vanilla extract was added to the tray beneath the floors to further distinguish the altered chambers from the training context.

Behavioral Procedure

Training and testing of contextual conditioning was performed as previously described (André, et al., 2008). Freezing, defined as the absence of all movement except respiration, was sampled for 1 s every 10 s and served as a measure of learning. During training, mice were placed into one of four conditioning chambers for 5.5 min. Baseline freezing behavior was recorded during the first 120 s of the session. At 148 s, mice were presented with a 2 s 0.57 mA foot shock US. At 298 s, an additional 2 s foot shock US was presented. The mice remained in the chambers for 30 s after the second US presentation. Approximately 24 hours later, testing of contextual conditioning occurred via placement of the mouse into the training context and freezing was scored for 5 min.

The 24 mg/kg/d nicotine dose was found to produce deficits in contextual conditioning after one day of treatment. To determine if this deficit was specific to contextual conditioning or due to an overall deficit in learning and memory, a separate group of mice underwent training and testing of delay fear conditioning. The behavioral procedure was performed as previously described (Gould & Higgins, 2003). Mice were placed into the training context and after a 120 s baseline period a 30 s auditory CS (85 dB white noise) was sounded that co-terminated with a 2 s US footshock (0.57 mA). After a 120 s ITI, another CS-US pairing was presented. Mice remained in the chambers for an additional 30 s after the second CS-US pairing. Approximately 24 hours later mice were placed back into the original training context without the CS for 5.5 min and freezing to the context freezing was scored for 5 min. Approximately 1 h later, mice were placed into the altered context for a total of 6 min. Generalized freezing was scored for

the first 3 min in the absence of the CS. The CS was then turned on and cued freezing was scored for 3 min.

Statistical Analyses

For studies examining the effects of chronic nicotine and withdrawal from chronic nicotine on contextual conditioning, data were analyzed using oneway analysis of variance (ANOVA). Significant omnibus tests were followed by Tukey's Honestly Significant Difference (HSD) tests. Games-Howell tests were used when the homogeneity of variance assumption was not satisfied. For the experiment using delay fear conditioning, independent samples t-tests were used to compare freezing levels within each condition. Any animal that was 2.5 standard deviations from the mean was considered an outlier and excluded from data analysis. This resulted in the removal of one mouse from data analysis.

Results

To determine when chronic nicotine enhances contextual conditioning at which doses, separate experiments were performed in which mice were implanted with osmotic minipumps that delivered chronic saline or nicotine (3, 6.3, 12, 24 mg/kg/d) for 1-6 days depending on the dose. Results revealed that different doses of nicotine enhanced contextual conditioning, or produced deficits in contextual conditioning, at different times. For 3 mg/kg/d nicotine (n = 9-12 per group), results revealed a significant effect on contextual conditioning, $F(5, 61) = 2.732$, $p < 0.05$ (Figure 1). However, this

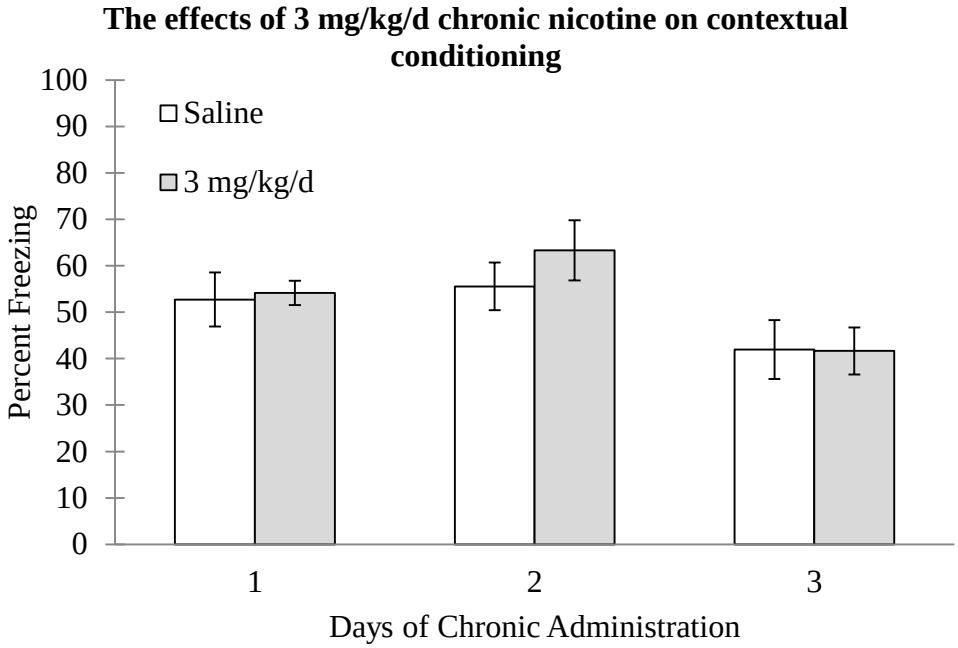

Figure 1. The effects of 3 mg/kg/d chronic nicotine on contextual conditioning. There was no significant effect of nicotine on contextual conditioning within each day. Error bars represent ± the standard error of the mean

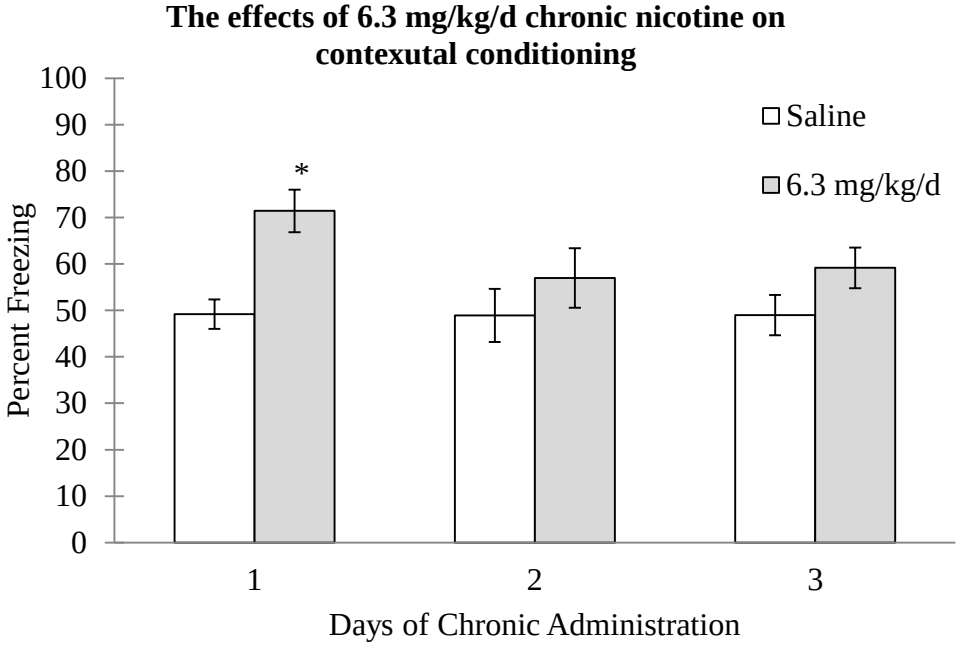

Figure 2. The effects of 6.3 mg/kg/d chronic nicotine on contextual conditioning. 6.3 mg/kg/d chronic nicotine enhanced contextual conditioning following 1 day of chronic administration. Error bars represent ± the standard error of the mean. (*) indicates $p < 0.05$ compared to saline treated mice within the same day

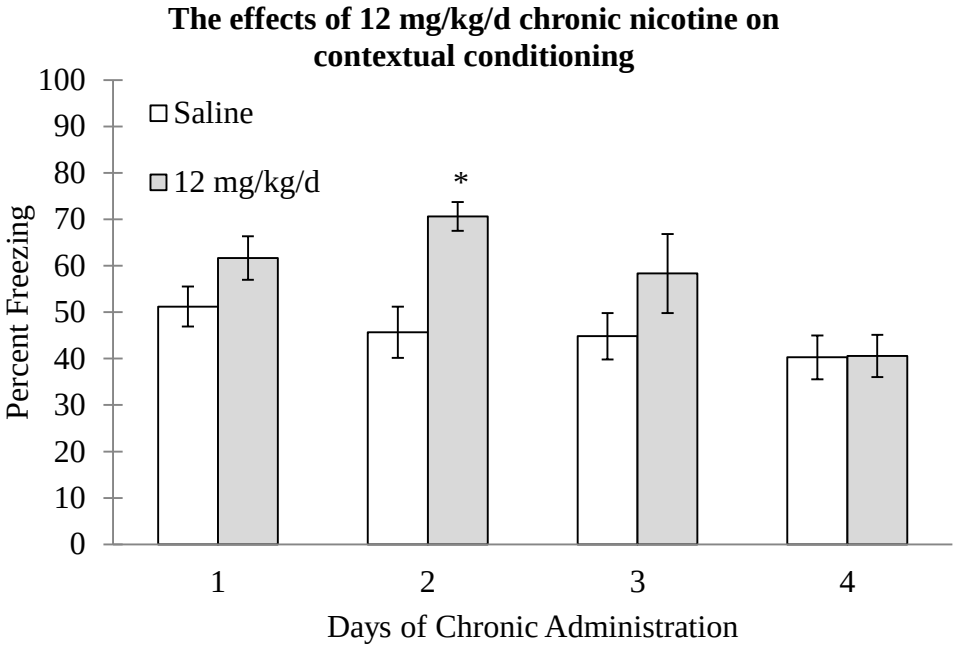

Figure 3. The effects of 12 mg/kg/d chronic nicotine on contextual conditioning. 12 mg/kg/d chronic nicotine enhanced contextual conditioning following 2 days of chronic administration. Error bars represent ± the standard error of the mean. (*) indicates $p < 0.05$ compared to saline treated mice within the same day

significant omnibus test was due to significant differences between animals that received 2 days of chronic nicotine and 3 days of chronic saline ($p < 0.05$) and 3 days of chronic nicotine ($p < 0.05$), comparisons that were not of interest. There were no significant differences between saline- or nicotine-treated animals within each day ($p > 0.05$). There was no significant effect of 3 mg/kg/d on baseline freezing ($p > 0.05$). Thus, 3 mg/kg/d did not enhance contextual conditioning at any day (1-3) tested.

There was a significant effect of 6.3 mg/kg/d (n = 9-14 per group) on contextual conditioning, $F(5, 62) = 4.125$, $p < 0.05$ (Figure 2). Post-hoc tests revealed that mice treated with 6.3 mg/kg/d for 1 day froze more to the context than mice treated with saline for 1 day ($p < 0.05$). There was no effect of saline or 6.3 mg/kg/d nicotine with 2-3 days

of chronic treatment ($p > 0.05$), which suggests the development of tolerance. In addition, there was no significant effect on baseline freezing ($p > 0.05$).

Likewise, there was a significant effect of 12 mg/kg/d (n = 9-12 per group) chronic nicotine treatment on contextual conditioning, $F(7, 80) = 4.758$, $p < 0.001$ (Figure 3). Games-Howell post- hoc tests revealed that mice treated with 12 mg/kg/d for 2 days froze more to the context than mice treated with saline for 2 days ($p < 0.05$). There was no significant effect of saline or 12 mg/kg/d nicotine on contextual freezing following 1, 3, or 4 days of chronic treatment ($p > 0.05$). In addition, there was no significant effect of 12 mg/kg/d on baseline freezing ($p > 0.05$).

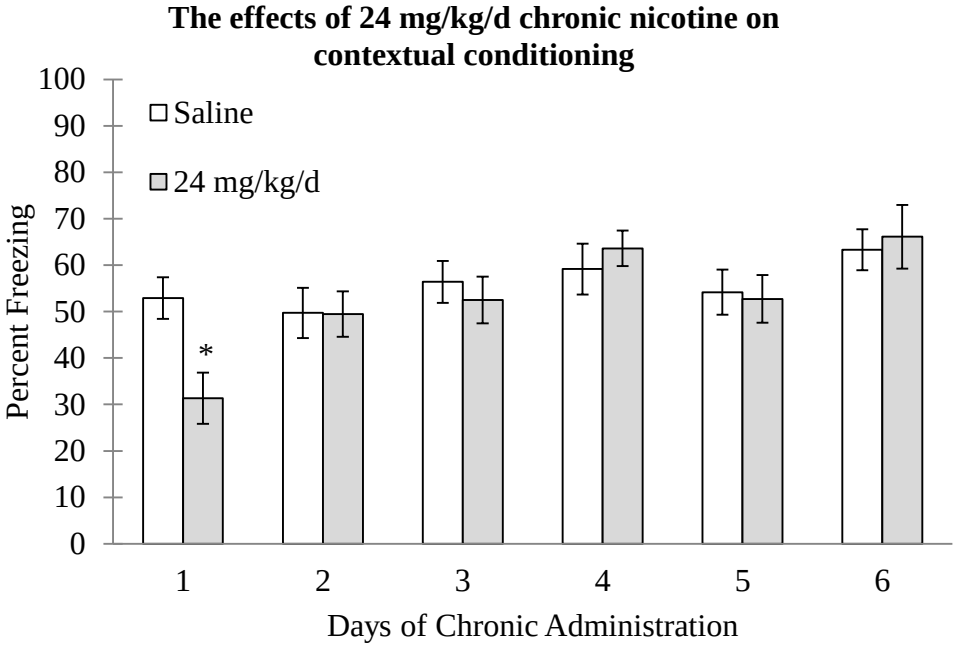

Figure 4. The effects of 24 mg/kg/d chronic nicotine on contextual conditioning. 24 mg/kg/d chronic nicotine produced a deficit in contextual conditioning following 1 day of chronic administration. Error bars represent ± the standard error of the mean. (*) indicates $p < 0.05$ compared to saline treated mice with the same day

Lastly, there was a significant effect of 24 mg/kg/d (n = 8-16 per group) chronic nicotine on contextual freezing, $F(11, 134) = 3.715$, $p < 0.001$ (Figure 4). Post-hoc tests

revealed that mice treated with 24 mg/kg/d for 1 day froze significantly less to the context than mice treated with chronic saline ($p < 0.05$). There was no effect of saline or 24 mg/kg/d nicotine on contextual freezing following 2-6 days of chronic treatment (all $ps > 0.05$). There was a significant effect of 24 mg/kg/d on baseline freezing, $F(11, 134) = 2.236$, $p < 0.05$. Games-Howell post-hoc tests did not reveal any significant differences between groups

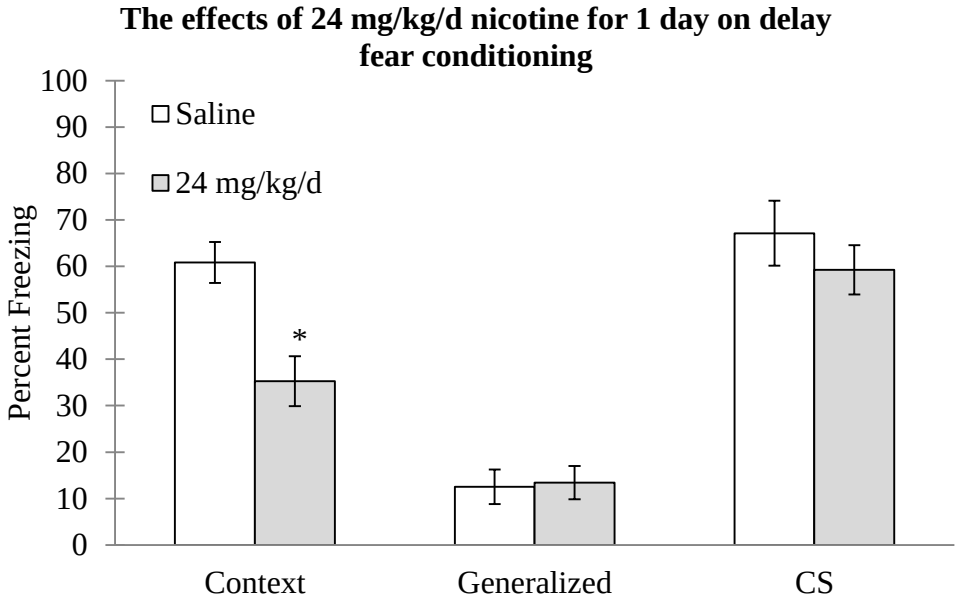

Figure 5. The effects of 24 mg/kg/d chronic nicotine for 1 day on delay fear conditioning. 24 mg/kg/d chronic nicotine produced a deficit in contextual conditioning following 1 day of chronic administration. Error bars represent ± the standard error of the mean. (*) indicates $p < 0.05$ compared to saline treated mice

during baseline freezing (all $ps > 0.05$). However, the significant effect of baseline was due to two animals each freezing once following 4 days of chronic nicotine treatment.

To determine if the deficit in contextual conditioning with 24 mg/kg/d for 1 day was specific to contextual conditioning or due to a global deficit in learning and memory, separate groups of mice (n = 12 per group) were trained in delay fear conditioning

(Figure 5). Independent samples t-tests revealed that mice administered 24 mg/kg/d chronic nicotine for 1 day froze less to the context than saline treated mice ($t(21) = 3.542$, $p < 0.05$). There was no significant effect of chronic nicotine or saline on generalized or cued freezing ($p > 0.05$).

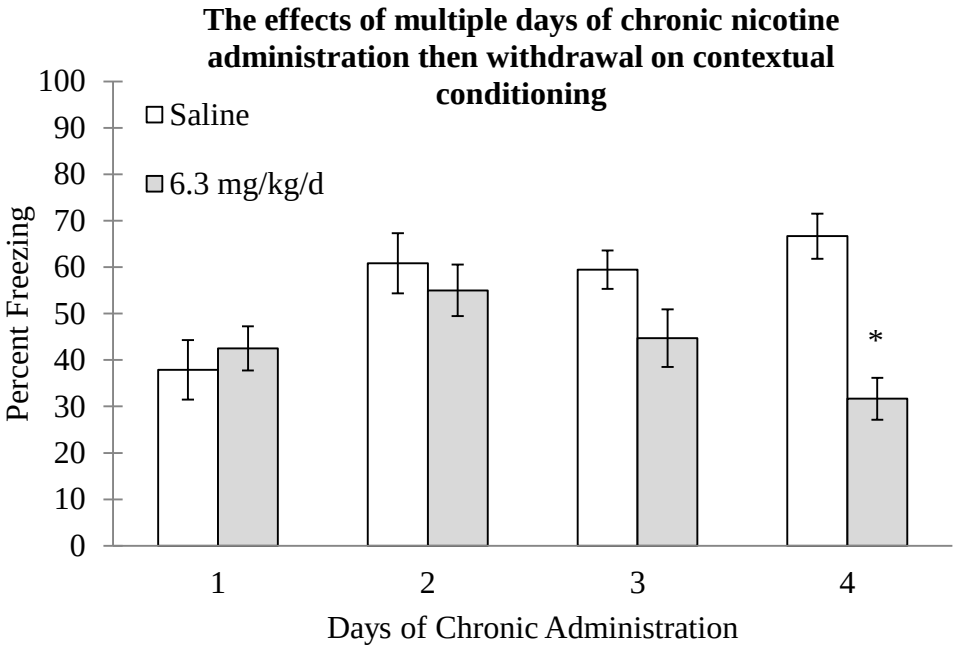

Figure 6. The effects of multiple days of chronic nicotine administration then withdrawal on contextual conditioning. 6.3 mg/kg/d chronic nicotine produced a deficit in contextual conditioning following withdrawal from 4 days of chronic nicotine administration. Error bars represent ± the standard error of the mean. (*) indicates $p < 0.05$ compared to saline treated mice within the same day

In order to determine the threshold of chronic nicotine treatment required to produce withdrawal-related deficits in contextual conditioning upon cessation of treatment, mice were administered chronic saline or 6.3 mg/kg/d nicotine for 1-4 days (n = 8-12 per group) then withdrawn from chronic treatment for 24 hours prior to training in contextual conditioning (Figure 6). A oneway ANOVA revealed a significant effect on contextual freezing, $F(7, 71) = 5.092$, $p < 0.001$. Post-hoc tests revealed that mice

withdrawn from 4 days of chronic nicotine froze significantly less to the context than mice withdrawn from 4 days of chronic saline ($p < 0.05$). There was no effect of baseline freezing ($p > 0.05$).

Discussion

The present study found that chronic nicotine enhanced contextual conditioning in a time- and dose-dependent manner and tolerance developed to the enhancing effect of chronic nicotine at different times with different doses (Summarized in Table 2). This is

Table 2. The effects of chronic nicotine on contextual conditioning

	Days of Administration					
Dose (mg/kg/d)	**1**	**2**	**3**	**4**	**5**	**6**
3	ø	ø	ø			
6.3	↑	ø	ø			
12	ø	↑	ø	ø		
24	↓	ø	ø	ø	ø	ø

Ø indicates no effect; ↓ indicates a deficit in contextual conditioning; ↑ indicates enhanced contextual conditioning

the first study to examine the effects of multiple doses and durations of chronic nicotine exposure on contextual conditioning. The lowest dose of nicotine (3 mg/kg/d) had no effect on contextual conditioning at any day tested. However, 6.3 mg/kg/d enhanced contextual conditioning after one day of chronic nicotine treatment but failed to alter contextual conditioning after two and three days of treatment, indicating that tolerance developed rapidly to the enhancing effect of chronic nicotine. In contrast, 12 mg/kg/d had no effect at one day, enhanced contextual conditioning at two days, and failed to

enhance after three and four days of chronic treatment demonstrating tolerance. Also, 24 mg/kg/d did not have an enhancing effect at any day tested but it produced a deficit in contextual conditioning after one day of treatment. This deficit was specific to hippocampus-dependent contextual conditioning as it did not affect cued conditioning, a hippocampus-independent learning and memory task (Logue, et al., 1997; Phillips & LeDoux, 1992). Thus, the ability of chronic nicotine to enhance contextual conditioning depends greatly not only on the dose administered but also the duration of treatment.

The present study also found that nicotine withdrawal deficits in contextual conditioning emerge after 4 days of 6.3 mg/kg/d chronic nicotine treatment. This suggests that chronic nicotine produces changes in hippocampal function after around 4 days of chronic nicotine exposure resulting in cognitive deficits upon cessation of treatment. The appearance of a withdrawal deficit in contextual conditioning after 4 days and the development of tolerance after 2 days suggests that tolerance and withdrawal are dissociable. It is plausible that the homeostatic response that resulted in the observation of tolerance also produced impairments in contextual conditioning during withdrawal. The process that pulled the system down to baseline levels resulting in tolerance after 2 days of administration was insufficient to produce withdrawal-related impairments when nicotine was removed, at this time point. However, as the duration of chronic nicotine increased, this homeostatic process gained enough strength over time, resulting in withdrawal-related impairments when nicotine was removed. By this account, tolerance and withdrawal are manifestations of the same process. However, this account is unlikely, because 6.3 mg/kg/d did not impair contextual conditioning after 3 days of administration. If the withdrawal deficit was a manifestation of the same process

involved in tolerance, that gained strength over time, then long durations of chronic nicotine exposure should impair contextual conditioning. In addition, even 13 days of chronic exposure with 6.3 mg/kg/d does not impair contextual conditioning (Davis, et al., 2005).

Although not explored in the present series of experiments, previous research suggests that the enhancing effect of chronic nicotine is mediated by the same receptors involved in the acute enhancing effect of nicotine. Acute, systemic administration of nicotine enhances contextual conditioning in α7, β3, and β4 KO mice, but not in β2 KO mice, indicating that nAChRs containing the β2 subunit are involved in the enhancing effects of acute nicotine on contextual conditioning (Davis, et al., 2007; Wehner et al., 2004). Likewise, systemic administration of the high-affinity receptor antagonist dihydro-beta-erythroidine (DHβE) blocks the enhancing effect of acute nicotine while systemic administration of the α7 antagonist methyllycaconitine (MLA) does not block the enhancing effect of nicotine on contextual conditioning (Davis & Gould, 2006). More specifically, infusions of DHβE but not MLA into the dorsal hippocampus block the enhancing effects of systemic nicotine (Davis, et al., 2007). Collectively, these studies indicate that high-affinity dorsal hippocampal β2-containing nAChRs, possibly α4β2* (* indicates other subunits may be incorporated), are involved in the enhancing effects of acute nicotine on contextual conditioning. Thus, it is likely that hippocampal α4β2* nAChRs are involved in the enhancing effect of chronic nicotine found in the present study.

These same receptors are also involved in the withdrawal effects of nicotine (Davis & Gould, 2009; Portugal, Kenney, & Gould, 2008). β2 KO mice do not exhibit

nicotine withdrawal deficits in contextual conditioning while α7 KO mice do (Portugal, et al., 2008). Mice withdrawn from chronic hippocampal infusions of nicotine show deficits in contextual conditioning compared to mice withdrawn from chronic hippocampal infusions of saline (Davis & Gould, 2009). In addition, C57BL/6, β2 KO, or wild-type mice treated with chronic systemic nicotine received intrahippocampal infusions of DHβE to precipitate withdrawal. The intrahippocampal infusions of DHβE produced deficits in contextual conditioning in C57BL/6 and wild-type mice, but not in β2 KO mice. These results indicate that hippocampal nAChRs containing the β2 subunit, possibly the α4β2* nAChR, mediate the deficits in contextual conditioning produced by withdrawal from chronic nicotine (Davis & Gould, 2009). Thus, it is very likely that changes in hippocampal β2-containing receptors, possibly the α4β2* nAChR, mediate the behavioral effects of chronic nicotine and withdrawal in contextual conditioning observed in the present study.

Although it is likely that the same receptors are involved in tolerance and withdrawal, different cellular responses of these receptors to chronic nicotine likely contribute to tolerance and withdrawal. The finding that tolerance develops to the enhancing effect of 6.3 mg/kg/d chronic nicotine after one day of administration and four days of chronic nicotine are required to impair contextual conditioning upon withdrawal suggests that tolerance and withdrawal are dissociable phenomenon, mediated by separate mechanisms, as the conditions to produce tolerance were not sufficient to produce withdrawal. The dual actions of nicotine in both desensitizing (Gentry & Lukas, 2002; Marks, et al., 1994; Ochoa, et al., 1992) and upregulating nAChRs (Govind, Vezina, & Green, 2009; Marks, et al., 1983; Marks, et al., 1985; Wonnacott, 1990),

especially α4β2* nAChRs (Flores, Rogers, Pabreza, Wolfe, & Kellar, 1992; McCallum, Collins, Paylor, & Marks, 2006), may underlie both the development of tolerance and withdrawal effects. Desensitized nAChRs lose function that would decrease behavioral responses mediated by nicotine (Marks, et al., 1994). This could be one mechanism that explains why 6.3 mg/kg/d first enhances contextual conditioning after 1 day of chronic treatment then fails to enhance with 2 and 3 days of chronic treatment. In support, rubidium efflux assays that measure nAChR function show that chronic nicotine decreases receptor function (Marks, et al., 1993).

The 12 mg/kg/d dose was initially too high to enhance contextual conditioning after one day of chronic nicotine exposure, a time period when 6.3 mg/kg/d enhanced contextual conditioning. This finding is in line with the inverted-U shaped dose-response curve produced by nicotine on many behavioral and cognitive functions including contextual conditioning (Gould & Higgins, 2003; Picciotto, 2003). As the duration of exposure increased, 12 mg/kg/d enhanced contextual conditioning on the second day of treatment, a time period when 6.3 mg/kg/d failed to alter contextual conditioning. Emergence of cognitive enhancement with chronic nicotine has previously been demonstrated in the radial arm maze (Levin, et al., 1990). It is likely that this dose not only desensitized nAChRs but also upregulated nAChRs. Some research groups have found reduced receptor function following chronic nicotine exposure (Fenster, Whitworth, Sheffield, Quick, & Lester, 1999; Marks, et al., 1993; Marks, et al., 1994), while other groups have shown increased function (Buisson & Bertrand, 2001; Nashmi et al., 2007; Nguyen, Rasmussen, & Perry, 2004). The increased receptor function is likely due to chronic nicotine upregulating functional receptors (Nguyen, et al., 2004), which

could lead to an overall gain of function, as is commonly observed with drug-induced receptor upregulation (Wonnacott, 1990). The end result of the combination of upregulation of functional receptors and desensitization could be a shift of the dose- and time-response curves for the ability of chronic nicotine to enhance contextual conditioning. The shift in balance between upregulation and desensitization resulted in 12 mg/kg/d producing a physiological state that was sufficient to enhance contextual conditioning on the 2nd day of treatment that was similar to that produced by 6.3 mg/kg/d on the 1st day of administration. Finally, the degree of upregulation and desensitization reached an equilibrium that resulted in tolerance to the enhancing effect of 12 mg/kg/d on the third day of administration.

Contrary to lower doses, which enhanced contextual conditioning, the results of the present study indicate that 24 mg/kg/d produced a deficit in learning and memory after 1 day of treatment. This deficit was specific to hippocampus-dependent learning and memory as there was no effect on cued conditioning, a type of hippocampus-independent learning and memory (Logue, et al., 1997; Phillips & LeDoux, 1992). The effective doses of nicotine in producing a behavioral response are generally within a narrow range, with lower doses producing no effect and higher doses having a disruptive effect (Picciotto, 2003). The finding that a high dose of nicotine disrupted hippocampal function is in line with previous research (Gould & Wehner, 1999).

Tolerance was also observed for the impairing effect of 24 mg/kg/d chronic nicotine, which is in line with previous reports of tolerance to the impairing effects of nicotine (Kenney, et al., 2011). It is possible that this high dose of nicotine over-activated the nAChR system resulting in a performance deficit, and then nAChRs became

desensitized and nullified this impairing effect. However, one would expect this dose to eventually enhance contextual conditioning through the same process that resulted in 12 mg/kg/d enhancing contextual conditioning on the 2nd day of treatment.

It is possible that enhancement would have occurred at time windows shorter than those tested in the present experiments (i.e., at 12 hour intervals rather than 24 hour intervals). This suggests that the process in which the shift in balance between desensitization and upregulation producing a physiological state sufficient to enhance contextual conditioning occurs at a different rate for 24 mg/kg/d than 12 mg/kg/d. In support, upregulation is dose-dependent, with higher concentrations of nicotine producing upregulation quicker than lower concentrations (Marks et al., 1983; Walsh et al., 2008). In addition, it is also possible that there are multiple mechanisms involved in tolerance, and different processes mediate tolerance to the enhancing and impairing effects of chronic nicotine. Tolerance to many of the effects of nicotine dissipate at different times, suggesting that different process mediate each effect (Marks, et al., 1985).

Likewise, it has recently been demonstrated that chronic nicotine-induced upregulation is the result of two processes caused by different mechanisms (Govind, Walsh, & Green, 2012). The first, initial process of upregulation was found to be characterized by a fast onset that rapidly reversed and corresponded to nAChRs transitioning from a low-affinity resting state to a high-affinity upregulated state, as previously proposed (Vallejo et al., 2005). The second process was characterized by a slow onset and offset that required longer exposure to nicotine to initiate than the first. This second, longer-lasting process was associated with changes in α4β2 number rather

than affinity and thus caused by a separate mechanism than the first. Although only one dose of nicotine was used to examine the two components of upregulation, it is possible that different doses of nicotine can initiate each process of upregulation as upregulation was dose-dependent (Govind et al., 2012).

The observation that nAChR upregulation is caused by multiple mechanisms suggests that these processes may contribute differently to consequences of chronic nicotine exposure such as withdrawal, sensitization, and tolerance. In relation to the results of the present study, it is possible that the 24 mg/kg/d dose initiated the second component of upregulation that was not initiated by lower doses. This second component of upregulation could have then contributed to the observation of tolerance to the impairing effect of nicotine and a lack of enhancement of contextual conditioning. Changes in nAChR number rather than affinity would likely have different behavioral consequences than changes in nAChR affinity alone.

Finally, when chronic nicotine is removed, nAChRs regain function while there are still an upregulated number of receptors. The excess number of receptors in the hippocampus could lead to a hypersensitive nAChR system, which could alter hippocampal function leading to withdrawal deficits in cognitive function. Findings from our laboratory indicate that upregulated receptors return to control level at a time course that parallels the disappearance of withdrawal-related impairments in contextual conditioning (Gould, et al., 2012). These parallel changes in upregulated high-affinity nAChRs and cognition suggest that nAChR upregulation contributes, in part, to the withdrawal effects during cessation of treatment.

In sum, the current series of experiments found that chronic nicotine enhanced contextual conditioning in a time- and dose-dependent manner. Tolerance developed to the enhancing effect of chronic nicotine as well as the disruptive effect of high doses of chronic nicotine on contextual conditioning. It was also found that chronic nicotine needs to be administered for 4 days before producing deficits in contextual conditioning from withdrawal. Tolerance developed to the enhancing effect of chronic nicotine at a different time point then withdrawal-deficits in contextual conditioning, suggesting separate underlying neural substrates. The enhancing and disruptive effects of chronic nicotine and withdrawal from chronic nicotine, respectively, are likely mediated by β2 containing nAChRs, possibly α4β2* nAChRs. The dual actions of chronic nicotine in both desensitizing and upregulating α4β2* nAChRs are likely to contribute to the current findings. Receptor desensitization likely resulted in tolerance to the cognitive enhancing effect of 6.3 mg/kg/d after 2 days of administration. The 12 mg/kg/d dose was initially too high to enhance contextual conditioning. However, as the duration of chronic nicotine treatment increased, receptor desensitization likely shifted the dose-response curve, producing a physiological state on the 2nd day of administration that was similar to 6.3 mg/kg/d enhancing contextual conditioning after 1 day of administration. In addition, receptor desensitization likely resulted in tolerance to the impairing effect of 24 mg/kg/d. Alternatively, it is possible that additional mechanisms contributed to tolerance to the impairing effect of 24 mg/kg/d.

The 6.3 mg/kg/d dose impaired contextual conditioning after 24 hours of withdrawal from 4 days of chronic nicotine administration. This dose not only desensitized but also upregulated nAChRs. When chronic nicotine was removed,

nAChRs regained function. The increased number of functional receptors led to a hypersensitive nAChR, which altered hippocampal function leading to withdrawal-deficits in contextual conditioning.

CHAPTER 3

SENSITIVITY TO ACUTE NICOTINE DURING NICOTINE WITHDRAWAL

The results of the studies in Chapter 2 demonstrated that tolerance to the enhancing effect of chronic nicotine and nicotine withdrawal-deficits in contextual conditioning emerge at different times, suggesting they are dissociable, and that nicotine-induced desensitization and upregulation of α4β2* nAChRs contribute to tolerance and withdrawal. It is likely then that these same mechanisms contribute to the state of the nAChRs system during withdrawal. Research indicates that the number of nAChRs remain increased for extended periods of time during withdrawal in regions important for learning and memory (Collins, Romm, & Wehner, 1988; Gould, et al., 2012; Marks, et al., 1985). Upregulated receptors return to baseline levels as the duration of withdrawal increases (Collins, et al., 1988; Gould, et al., 2012; Marks, et al., 1985). Data from our laboratory show that chronic nicotine increases the number of high-affinity nAChRs in the hippocampus, which then decrease as a function of time and match withdrawal-related deficits in contextual conditioning (Gould, et al., 2012). During this withdrawal period, it is likely that nAChRs also become resensitized (Dani & Heinemann, 1996; Gould, et al., 2012). The recovery of nAChR function during withdrawal coupled with an increased density of receptors may contribute to a hyperfunctional nAChR system during withdrawal. Indeed, as previously suggested, because of the increased number of nAChRs that are responsive during withdrawal, some cholinergic systems become hyperexcitable (Dani & Heinemann, 1996).

A hyperfunctional nAChR system suggests that there might be enhanced sensitivity to acute nicotine and other nicotinic agonists during a period of nicotine

withdrawal. There have been relatively few studies that have examined the effects of acute nicotine on behavioral and physiological function during a period of withdrawal. However, multiple studies have demonstrated sensitized behavioral and physiological responses to acute nicotine following exposure to repeated injections of acute nicotine. Locomotor sensitization has been used as a measure of sensitivity to acute nicotine following repeated injections (Benwell & Balfour, 1992; Domino, 2001; Stolerman, Fink, & Jarvik, 1973). Usually the first dose of nicotine reduces locomotor activity but tolerance to this effect develops rapidly (Clarke & Kumar, 1983). Soon after, subsequent doses of nicotine lead to greater locomotor activity. Animals usually receive daily or twice-daily injections of nicotine in their home cages or testing apparatus and then on subsequent days locomotor sensitization is tested following a single injection of nicotine. Using procedures such as these, Domino (2001) found that after 5 twice-daily injections of 0.32 mg/kg nicotine, rats had enhanced locomotor activity during a testing period in response to an acute challenge dose of 0.32 mg/kg nicotine. In addition, daily pretreatment with 0.1 or 0.4 mg/kg nicotine daily for 5 days enhanced locomotor activity on the 6^{th} day when given acute challenge doses of 0.1 or 0.4 mg/kg nicotine (Benwell & Balfour, 1992). Thus, prior exposure to nicotine results in sensitized locomotor responses to acute nicotine.

Additional evidence for enhanced sensitivity to acute nicotine following nicotine administration comes from studies of neurotransmitter release and *in vitro* studies of nAChR function. Neurotransmitter release is enhanced after exposure to an acute challenge dose of nicotine following multiple nicotine injections. For example, twice-daily injections of 0.4 mg/kg nicotine for 4 days increased cortical acetylcholine release

when tested on the 5th day following a single injection of 0.4 mg/kg nicotine (Arnold, Nelson, Sarter, & Bruno, 2003). In addition, dopamine release in the nucleus accumbens is enhanced following chronic nicotine administration (Balfour, Benwell, Birrell, Kelly, & Al-Aloul, 1998; Benwell & Balfour, 1992; Rahman, Zhang, & Corrigall, 2003). *In vitro* studies provide more evidence for enhanced sensitivity during withdrawal. Human α4β2 nAChRs expressed in HEK-239 cells can be activated in the presence of chronic nicotine and, after nicotine removal, α4β2 nAChRs display signs of hyperfunctionality as demonstrated by a higher affinity for acetylcholine, currents of higher amplitudes, and less evidence of desensitization (Buisson & Bertrand, 2001). Likewise, chronic nicotine also produces changes in hippocampal excitability that might be a result of a hyperfunctional nAChR system. In hippocampal slices from rats treated with chronic nicotine for 1 week, withdrawal from nicotine produces an increase in hippocampal CA1 pyramidal cell excitability that persists up to 9 months (Penton, Quick, & Lester, 2011). Together, these findings along with the behavioral evidence suggest that the nAChR system is hyperfunctional following repeated exposure to nicotine.

In the behavioral studies discussed above, nicotine was administered through multiple daily injections. Multiple injection paradigms have the advantage that the dose and time of administration are well controlled. In addition, given the relatively short half-life of nicotine in the rat (45 minutes versus 2 hours in humans (Matta et al., 2007)), nicotine is cleared before each injection resulting in nAChR activation with each dose. However, it is unclear if this pattern of nicotine administration is representative of chronic administration observed in smokers. Smokers will adjust their level of cigarette smoking, maintaining relatively constant plasma nicotine levels (Henningfield &

Goldberg, 1988). Smoking in this manner results in a near complete saturation and desensitization of nAChRs (Benowitz, 2008; Brody et al., 2006), rather than periods of activation, desensitization, and resensitization as with multiple injections. To address these methodological considerations, researchers frequently utilize the osmotic minipump as a means of administering nicotine chronically. Continuous nicotine infusion through the minipump allows steady-state plasma levels to be achieved similar to human smoking. In addition, osmotic minipumps have the additional benefit of being able to be removed in order to examine spontaneous nicotine withdrawal. Therefore, studies that utilize osmotic minipumps are crucial to understanding sensitivity to nicotine during a period of nicotine withdrawal. Using procedures such as these, Benwell, Balfour, and Birrell (1995) found that constant infusion of nicotine through subcutaneously implanted osmotic minipumps nearly abolished the sensitized locomotor response and dopamine response in the nucleus accumbens to acute injections of nicotine, suggesting desensitization of nAChRs. However, after pump removal, which induced spontaneous nicotine withdrawal, acute injections of nicotine resulted in a sensitized dopamine response in the nucleus accumbens and enhanced locomotor activity compared with responses obtained during constant nicotine infusion (Benwell, Balfour, & Birrell, 1995). Unfortunately, a withdrawal from chronic saline group was not included in this study to compare to rats withdrawn from chronic nicotine; therefore it is difficult to determine if there was indeed enhanced sensitivity to acute nicotine during a period of nicotine withdrawal or prior exposure to nicotine.

A recent study provides more evidence for enhanced sensitivity to nicotine during a period of nicotine withdrawal (Zhang, Dong, Doyon, & Dani, 2011). Mice were

administered nicotine (plus saccharin) chronically through their drinking water for 4 or 12 weeks, achieving levels of nicotine comparable to chronic smokers, and then were withdrawn from chronic nicotine treatment for 1 day. Nicotine withdrawal decreased basal dopamine levels in the medial nucleus accumbens compared to animals that received chronic saccharin as measured by *in vivo* microdialysis. During this withdrawal period, acute nicotine increased dopamine concentrations across all treatment groups. However, because chronic nicotine treated mice had lower baseline dopamine concentrations than chronic saccharin treated mice, data were normalized to baseline levels in order to examine the relative change in dopamine concentration. Analyzed this way, both 4 and 12 week nicotine withdrawal groups showed a significantly higher nicotine induced dopamine response than chronic saccharin treated mice (Zhang, et al., 2011). The authors suggest that the enhanced dopamine signaling in the nucleus accumbens, an area implicated in the reinforcing effects of multiple drugs of abuse, might render smokers experiencing withdrawal more vulnerable to reinforcing effects of nicotine.

Contextual conditioning serves as a good model to determine nAChRs are hypersensitive during withdrawal. Mice that are withdrawn from chronic nicotine show impaired contextual conditioning (Davis, et al., 2005). During nicotine withdrawal, administration of acute nicotine not only reverses withdrawal-related deficits in contextual conditioning but also enhances contextual conditioning (Davis, et al., 2005). As nicotine acts within the dorsal hippocampus to enhance contextual conditioning (Davis, et al., 2007), these data suggest that nAChRs in the hippocampus are hypersensitive during withdrawal. However, Davis et al. (2005) did not compare nicotine

enhancement to animals that were no longer in a state of nicotine withdrawal and only tested one dose. Therefore, it is difficult to determine if the enhanced sensitivity was due to a hypersensitive nAChR system during withdrawal, as suggested by Chapter 2, or due to prior exposure of nicotine. Therefore, the present study assessed the effects of acute nicotine on contextual conditioning immediately after cessation of treatment and two weeks later, a time period previously shown not to be associated with withdrawal deficits in learning (Gould, et al., 2012). This comparison should inform on whether there is indeed a hypersensitive nAChR system during withdrawal or if prior nicotine exposure produces long-lasting sensitization.

Method

Subjects

Subjects were male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) aged 8-12 weeks at the beginning of pump implantation. Mice were housed 1-4 per cage with *ad libitum* access to food and water. A 12-hour light/dark cycle was maintained from 7:00 AM to 7:00 PM with all experiments conducted during the light cycle. The Temple University Institutional Animal Care and Use Committee approve all experimental procedures.

Surgery

Mice were implanted with subcutaneous osmotic minipumps (Alzet, Model 1002, Durect Co, Cupertino, CA) that delivered chronic saline or nicotine at a rate of 0.25 µl/hr for 12 days. Osmotic minipumps were surgically inserted subcutaneously via an incision

in the lower back of the mouse. Surgery was performed under sterile conditions with 5% isoflurane as the anesthetic. A second, similar surgery was performed to remove pumps and induce spontaneous nicotine withdrawal (WCN) or saline withdrawal (WCS) 12 days after pump implantation.

Drugs

Nicotine hydrogen tartrate salt (Sigma, St. Louis, MO) was dissolved in 0.9% saline and administered intraperitoneally 2-4 minutes prior to training and testing of contextual conditioning. Doses were saline, 0.022, 0.045, 0.09, 0.18, or 0.36 mg/kg nicotine. Osmotic minipumps were filled with a 100 µl of solution that contained saline or 6.3 mg/kg/d nicotine. Doses reported as the freebase weight of nicotine and based on previous work (Davis, et al., 2005; Gould & Higgins, 2003; Gould & Wehner, 1999).

Experiments and Conditions

Separate groups of mice received chronic saline or nicotine for 12 days then were withdrawn from chronic treatment for either 24 hours (nicotine withdrawal) or 14 days (prior nicotine exposure). Each time point (24 hours or 14 days) had a saline and a nicotine withdrawal group. The 24 hour time point was chosen as the withdrawal time point because previous work indicates that 24 hours of withdrawal from 12 days of chronic nicotine treatment is sufficient to impair contextual conditioning (Davis, et al., 2005). Recent data from our laboratory indicate that upregulated high-affinity receptors in the hippocampus return to control levels after 5 days of withdrawal from 12 days of chronic nicotine treatment and withdrawal deficits dissipate at this time as well (Gould, et

al., 2012). Therefore, 14 days was chosen as the time point to compare to 24 hours of withdrawal to avoid any confounding effects of receptor upregulation or withdrawal-related impairments in contextual conditioning. After the withdrawal period, mice then received acute injections of saline (control groups) or 0.022, 0.045, 0.09, 0.18, 0.36 mg/kg. This resulted in 6 separate WCS and WCN groups within each time point.

Apparatus

Mice were trained and tested for contextual conditioning in four identical clear Plexiglas chambers (26.5 x 20.4 x 20.8 cm) housed in sound attenuating boxes (Med-Associates, St. Albans, VT). The floor of each chamber was made of metal bars (0.20 cm diameter) spaced 1.0 cm apart and connected to a shock generator and scrambler (Med Associates, Model ENV-414). Ventilation fans were mounted on the sides of each box to provide background noise. A 4 W light mounted above each box provided illumination. Stimulus administration was controlled by a PC running LabView software.

Behavioral Procedure

Training and testing of contextual conditioning was performed as previously described (André, et al., 2008). Freezing, defined as the absence of all movement except respiration, was sampled every for 1 s every 10 s and served as a measure of learning. During training mice, were placed into one of four conditioning chambers for 5.5 min. Baseline freezing behavior was recorded during the first 120 s of the session. At 148 s, mice were presented with a 2 s 0.57 mA foot shock US. At 298 s, an additional 2 s foot shock US was presented. The mice remained in the chambers for 30 s after the second US

presentation. Approximately 24 hours later, testing of contextual conditioning occurred via placement of the mice into the training contexts and freezing was scored for 5 min.

Statistical Analyses

Freezing was analyzed using oneway ANOVAs. To explore if there was enhanced sensitivity to acute nicotine during withdrawal, data were converted to percent change of control (control being WCS + saline or WCN + saline within each time point) then analyzed using a three way ANOVA followed by Bonferroni corrected planned contrasts. Significant omnibus tests and contrasts were followed by Tukey's HSD tests. Games-Howell post-hoc tests were used when the homogeneity of variance assumption was not satisfied. Any animal that was 2.5 standard deviations from the mean was considered an outlier and excluded from data analysis

Results

To determine the effects of 24 hours of nicotine withdrawal on the acute nicotine enhancement of contextual conditioning, mice were implanted with osmotic minipumps that delivered chronic saline or 6.3 mg/kg/d nicotine for 12 days. On day 12 pumps were removed to induce spontaneous withdrawal. After 24 hours of withdrawal, mice were trained and tested in contextual conditioning following injections of saline or nicotine (0.022, 0.045, 0.09, 0.18, or 0.36 mg/kg) at training and testing (n = 10-16 per group). A oneway ANOVA revealed a significant main effect of acute nicotine on baseline freezing, $F(11, 146) = 14.049$, $p < 0.001$ (Figure 7). Games-Howell post-hoc tests did

not reveal any significant differences between groups in baseline freezing that lead to a significant omnibus test.

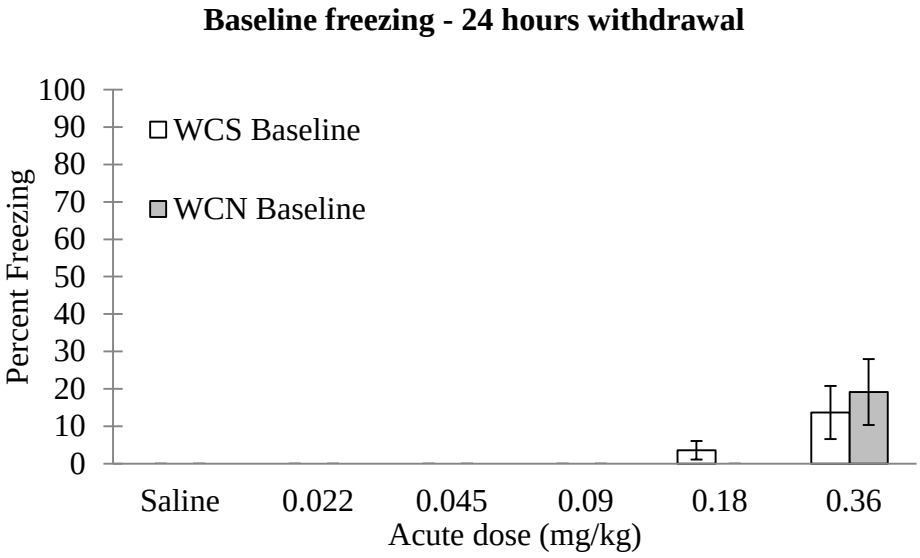

Figure 7. The effects of acute nicotine on baseline freezing 24 hours after 12 days of chronic nicotine treatment. 0.36 mg/kg increased baseline freezing across withdrawal groups. WCS = withdrawal from chronic saline. WCN = withdrawal from chronic nicotine. Error bars represent ± the standard error of the mean

However, visual inspection of the data show that mice in the WCS + 0.36 mg/kg nicotine and WCN + 0.36 mg/kg groups nicotine froze more to the apparatus than all other groups. There were no significant differences between these groups in baseline freezing ($p > 0.05$).

For contextual freezing, a oneway ANOVA revealed a significant main effect of acute nicotine on contextual freezing, $F(11, 146) = 5.288$, $p < 0.001$ (Figure 8). Games-Howell post-hoc tests revealed that mice undergoing withdrawal from chronic saline and administered 0.09 mg/kg ($p < 0.05$) and 0.36 mg/kg ($p < 0.001$) nicotine froze significantly more to the context than the WCS + Saline group. There was a trend toward enhancement in mice receiving 0.022 mg/kg ($p = 0.161$) and 0.045 mg/kg ($p = 0.089$)

nicotine. The WCN + Saline group froze significantly less to the context than the WCS + Saline group ($p < 0.05$) indicating that nicotine withdrawal disrupted contextual

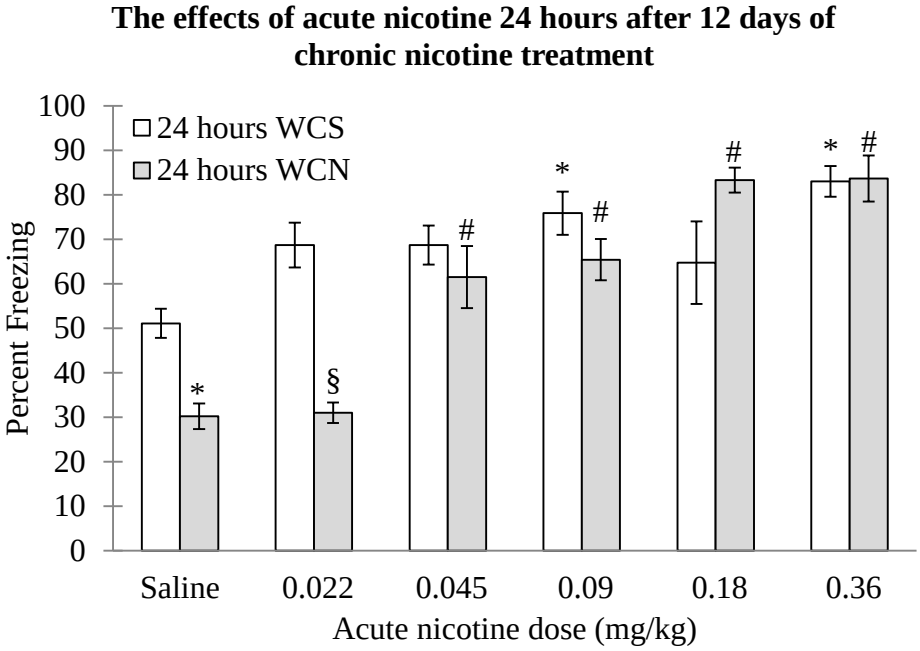

Figure 8. The effects of acute nicotine on contextual conditioning 24 hours after 12 days of chronic nicotine treatment. Nicotine enhanced contextual conditioning in animals that received chronic saline and chronic nicotine. WCS = withdrawal from chronic saline. WCN = withdrawal from chronic nicotine. Error bars represent ± the standard error of the mean. (*) indicates $p < 0.05$ compared to WCS + Saline treated mice. (#) indicates $p < 0.05$ compared to WCN + Saline treated mice. (§) indicates $p < 0.05$ compared to the WCS + 0.022 mg/kg group

conditioning and replicated previous findings (André, et al., 2008; Davis & Gould, 2007; Davis & Gould, 2009; Davis, et al., 2005; Portugal & Gould, 2007; Portugal, et al., 2008; Raybuck, et al., 2008; Wilkinson & Gould, 2011). Games-Howell post-hoc tests showed that mice that underwent nicotine withdrawal and received 0.045 mg/kg ($p < 0.05$), 0.09 mg/kg ($p < 0.05$), 0.18 mg/kg ($p < 0.05$), and 0.36 mg/kg ($p < 0.05$) nicotine froze more to the context than WCN + Saline mice. There were no significant differences between the WCN + Saline and WCN + 0.022 mg/kg groups ($p = 1$). The only major difference

between doses across withdrawal groups was at 0.022 mg/kg whereby WCS + 0.022 mg/kg froze more to the context than WCN + 0.022 mg/kg ($p < 0.001$).

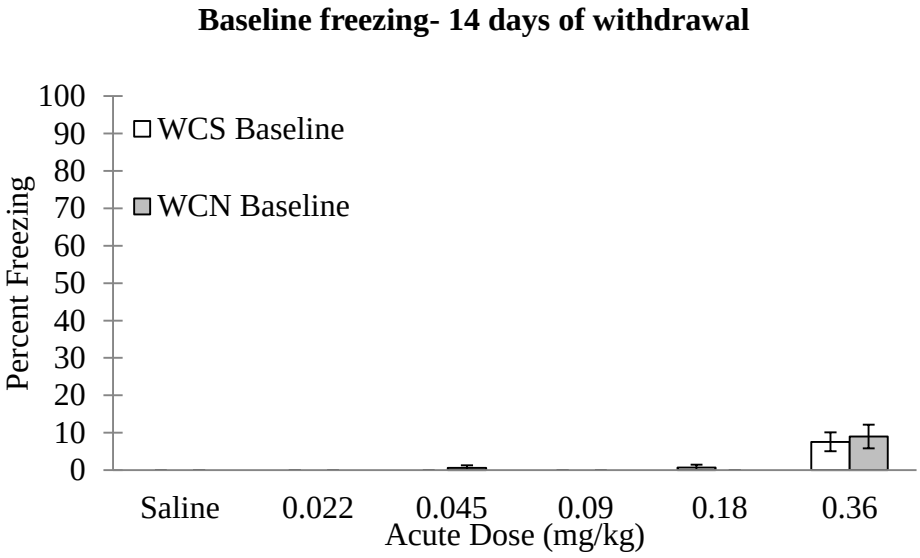

Figure 9. The effects of acute nicotine on baseline 14 days after 12 days of chronic nicotine treatment. 0.36 mg/kg increased baseline freezing across withdrawal groups. WCS = withdrawal from chronic saline. WCN = withdrawal from chronic nicotine. Error bars represent ± the standard error of the mean

To determine the effects of prior chronic nicotine exposure on the acute nicotine enhancement of contextual conditioning, mice were implanted with osmotic minipumps that delivered chronic saline or 6.3 mg/kg/d nicotine for 12 days. On day 12 pumps were removed to induce nicotine withdrawal. After 14 days of withdrawal, mice were trained and tested in contextual conditioning following injections of saline or nicotine (0.022, 0.045, 0.09, 0.18, or 0.36 mg/kg) at training and testing (n = 11-16 per group). A oneway ANOVA revealed a significant effect of acute nicotine on baseline freezing, $F(11, 146) = 8.402$, $p < 0.001$ (Figure 9). Games-Howell post-hoc tests determined that there were no specific differences between groups to produce the baseline effect. However, visual inspection of the baseline data show that the WCN + 0.36 mg/kg nicotine and WCS +

0.36 mg/kg nicotine groups froze more to the apparatus than all other groups. There were no differences between these two groups in baseline freezing ($p > 0.05$).

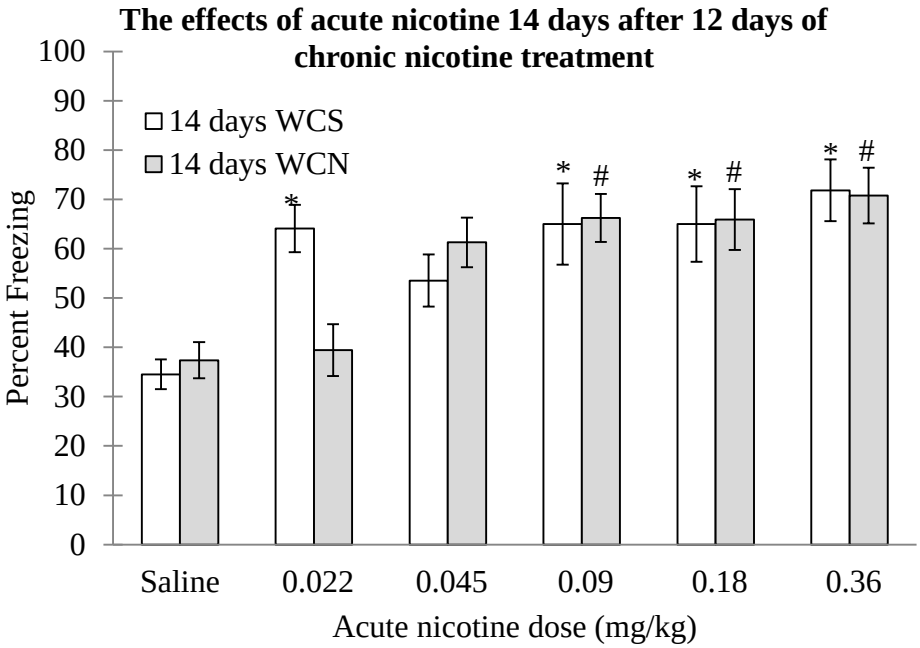

Figure 10. The effects of acute nicotine 14 days after 12 days of chronic nicotine treatment. Nicotine enhanced contextual conditioning in animals that received withdrawal from chronic saline and chronic nicotine. WCS = withdrawal from chronic saline. WCN = withdrawal from chronic nicotine. Error bars represent ± the standard error of the mean. (*) indicates $p < 0.05$ compared to WCS + Saline treated mice. (#) indicates $p < 0.05$ compared to WCN + Saline treated mice

For contextual conditioning, a oneway ANOVA revealed a significant effect of acute nicotine on contextual freezing, $F(11, 146) = 6.327$, $p < 0.001$ (Figure 10). Tukey's post-hoc tests revealed that animals receiving WCS + 0.022 mg/kg ($p < 0.05$), WCS + 0.09 mg/kg ($p < 0.05$), WCS + 0.18 mg/kg ($p < 0.05$), and WCS + 0.36 mg/kg ($p < 0.001$) nicotine froze significantly more to the context than animals receiving WCS + Saline. In a similar fashion, animals receiving WCN + 0.09 mg/kg ($p < 0.05$), WCN + 0.18 mg/kg ($p < 0.05$), and WCN + 0.36 mg/kg ($p < 0.05$) nicotine had enhanced freezing relative to the WCN + Saline group. There was a trend towards enhancement with WCN

+ 0.045 mg/kg ($p = 0.068$). There were no significant differences between any acute doses in animals receiving WCS or WCN (all $ps > 0.05$). Although 0.022 mg/kg enhanced contextual conditioning in the WCS group, it had no effect in the WCN group. There was a trend towards significance between WCS + 0.022 mg/kg and WCN + 0.022 mg/kg nicotine ($p = 0.072$).

To determine if there was enhanced sensitivity to acute nicotine during 24 hours of nicotine withdrawal, data were transformed into percent change from control, based on previous analyses of similar data (Zhang, et al., 2011), and then analyzed using a three way ANOVA (Figure 11). A three way ANOVA revealed a significant acute dose X time point X withdrawal from chronic treatment interaction, $F(4, 239) = 2.700$, $p < 0.05$. Bonferroni corrected planned contrasts revealed that mice withdrawn from chronic saline for 24 hours and administered acute nicotine had a smaller percent change from control than animals withdrawn from chronic saline for 14 days and administered acute nicotine ($t(76.061) = -4.600$, $p < 0.001$). This indicates that, on average, acute nicotine produced a greater enhancement of contextual conditioning in mice withdrawn from chronic saline for 14 days than withdrawn for 24 hours. On the other hand, this trend was reversed in mice withdrawn from chronic nicotine. Mice withdrawn from chronic nicotine for 24 hours and administered acute nicotine had a greater percent change from control than mice withdrawn from chronic nicotine for 14 days then administered acute nicotine ($t(89.771) = 5.734$, $p < 0.001$). This reversal of trend from animals withdrawn from chronic saline suggests that there is enhanced sensitivity to acute nicotine in animals withdrawn from chronic nicotine for 24 hours. Overall, mice withdrawn from chronic

nicotine for 24 hours then administered acute nicotine showed a greater percent change from control than all other withdrawal groups ($t(65.165) = 6.763$, $p < 0.001$).

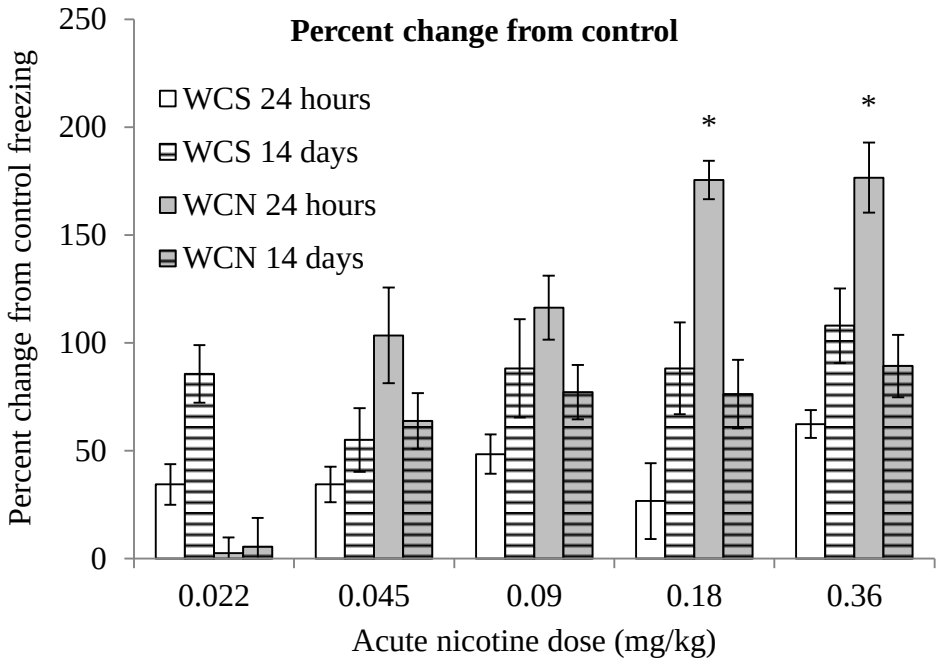

Figure 11. Combined acute contextual conditioning data converted to percent change from control. WCS = withdrawal from chronic saline. WCN = withdrawal from chronic nicotine. Error bars represent ± the standard error of the mean. (*) indicates $p < 0.05$ compared to withdrawal groups within the same acute dose

To further analyze sensitivity to acute nicotine during 24 hours of nicotine withdrawal, Bonferroni corrected planned contrasts were run to test the withdrawal from chronic treatment X time point interaction within each acute dose. There was a significant chronic X time point interaction within 0.18 mg/kg acute nicotine, $t(37.566) = -4.870$, $p < 0.001$. Games-Howell post-hoc tests revealed that mice withdrawn from chronic nicotine for 24 hours had a greater percent change from control than mice in other withdrawal groups (all $ps < 0.05$). In addition, there was a significant chronic X time point interaction within 0.36 mg/kg acute nicotine, $t(32.306) = -4.644$, $p < 0.001$. Tukey's post-hoc tests revealed that mice withdrawn from chronic nicotine for 24 hours

had a greater percent change from control than mice in other withdrawal groups (all ps < 0.05). Although there was a greater percent change from control in mice withdrawn from chronic nicotine for 24 hours then administered 0.045 and 0.09 mg/kg acute nicotine, these contrasts did not reach statistical significance with the Bonferroni correction.

Discussion

The present study provides evidence that the nAChR system is hypersensitive during a period of nicotine withdrawal. When mice were treated for 12 days of chronic saline and then tested 24 hours later in contextual conditioning following acute injections of saline or nicotine, two doses of nicotine, 0.09 and 0.36 mg/kg, enhanced contextual conditioning while there was a trend towards enhancement with 0.022 and 0.045 mg/kg. Withdrawal from chronic nicotine impaired contextual conditioning in saline injected mice, replicating previous findings that nicotine withdrawal impairs contextual conditioning (André, et al., 2008; Davis & Gould, 2007; Davis & Gould, 2009; Davis, et al., 2005; Portugal & Gould, 2007; Portugal, et al., 2008; Raybuck, et al., 2008; Wilkinson & Gould, 2011), and in mice that received 0.022 mg/kg nicotine. In addition, in nicotine withdrawn mice, all doses except 0.022 mg/kg enhanced contextual conditioning, suggesting sensitization. When mice were treated for 12 days of chronic saline then tested 14 days later in contextual conditioning, 0.022 mg/kg, 0.09 mg/kg, 0.18, and 0.36 mg/kg nicotine enhanced contextual conditioning. The only ineffective dose was 0.045 mg/kg. In the WCN group, 0.09 mg/kg, 0.18 mg/kg, and 0.36 mg/kg nicotine enhanced contextual conditioning. The doses that were ineffective were 0.022 mg/kg and 0.045 mg/kg nicotine. There was no difference between WCS mice injected

with saline and WCN mice injected with saline indicating no effect of prior chronic nicotine exposure on contextual conditioning. These findings are summarized in Table 3.

Table 3. Summary of the findings from Chapter 3

	24 Hours Withdrawal		14 Days Withdrawal	
Acute Dose (mg/kg)	**Saline**	**Nicotine**	**Saline**	**Nicotine**
Saline	ø	↓	ø	ø
0.022	ø	↓	↑	ø
0.045	ø	↑	ø	ø
0.09	↑	↑	↑	↑
0.18	ø	↑	↑	↑
0.36	↑	↑	↑	↑

Ø indicates no effect; ↓ indicates a deficit in contextual conditioning; ↑ indicates enhanced contextual conditioning

To analyze sensitivity to acute nicotine during nicotine withdrawal, data were transformed into percent change from control. Mice that underwent 14 days of withdrawal from chronic saline and administered acute nicotine had a greater percent change from control than mice that underwent 24 hours of withdrawal from chronic saline. This difference in percent change from control was most likely due to the time of recovery from the surgical procedures, which might affect learning. However, this trend was reversed in animals withdrawn from chronic nicotine. Mice that underwent 14 days of withdrawal from chronic nicotine and administered acute nicotine had a smaller percent change from control than mice that underwent 24 hours of withdrawal from chronic nicotine. This reversal suggests that there was enhanced sensitivity to acute nicotine during a period of nicotine withdrawal. Likewise, when compared to all other withdrawal groups across each acute nicotine dose, mice in the withdrawal from chronic

nicotine for 24 hours group showed a greater percent change from control than mice in all other withdrawal groups. Analysis of percent change from control within each acute dose further confirmed enhanced sensitivity to acute nicotine during nicotine withdrawal. Mice withdrawn from chronic nicotine for 24 hours and administered two out of the five acute nicotine doses (0.18, and 0.36 mg/kg) had a significantly greater percent change from control than all other withdrawal groups. Taken together, these data provide strong evidence that there is enhanced sensitivity to acute nicotine during a period of nicotine withdrawal and support previous suggestions that there is hyperexcitability of cholinergic systems during nicotine withdrawal (Dani & Heinemann, 1996).

There are a couple, although not mutually exclusive, mechanisms through which withdrawal from chronic nicotine might result in a hypersensitive nAChR system. First, as suggested in Chapter 2, nAChR number increases and function decreases during chronic nicotine treatment. When nicotine is removed, the excess nAChRs recover function. The recovery of receptor function coupled with the increased number of receptors may result in an overall gain of function in the nAChR system resulting in enhanced sensitivity to acute nicotine in addition to withdrawal-deficits in contextual conditioning. Second, chronic nicotine might produce changes in nAChR stoichiometry that leave them hypersensitive. Heteromeric neuronal nicotinic receptors are composed of combination of α (α2-α10) and β (β2-β4) subunits: two α subunits define the main binding structure; two complimentary non-α subunits; and a fifth subunit that is not directly involved in agonist binding but confers different properties to each receptor (Mineur & Picciotto, 2008). This allows for considerable molecular and functional diversity between nAChRs composed of similar subunits (Gotti et al., 2009). Inclusion of

the β2 subunit is a requirement for nAChR upregulation with chronic nicotine (McCallum, et al., 2006). Studies have shown that exposure to chronic nicotine results in an upregulation of α4β2 nAChRs that have a higher affinity and sensitivity to nicotinic agonists with less desensitization (Buisson & Bertrand, 2001; Vallejo, Buisson, Bertrand, & Green, 2005). This may be due to chronic nicotine favoring the formation of α4β2 nAChRs with a stoichiometry of $(\alpha4)_2(\beta2)_3$, which are more sensitive and desensitize slower than receptors with a stoichiometry of $(\alpha4)_3(\beta2)_2$ (Nelson, Kuryatov, Choi, Zhou, & Lindstrom, 2003). Thus, it is likely that long-term exposure to nicotine results in the formation of functional nAChRs that are more sensitive to nicotine. The greater sensitivity to nicotine is likely to contribute to the greater percent increase in freezing observed in mice withdrawn from chronic nicotine compared to saline withdrawn mice exposed to acute nicotine.

One finding that speaks against the suggestion of a hypersensitive nAChR system during nicotine withdrawal is that 0.022 mg/kg did not enhance contextual conditioning in nicotine withdrawn mice at either time point. A hypersensitive nAChR system should show enhancement at this low dose during 24 hours of nicotine withdrawal. One possibility for this discrepancy might be that chronic nicotine produces a mixed population of α4β2 nAChRs that have differing sensitivity to acute nicotine. The suggestion that chronic nicotine favors the formation of $(\alpha4)_2(\beta2)_3$ that are more sensitive and desensitize slower than receptors with a stoichiometry of $(\alpha4)_3(\beta2)_2$ (Kuryatov, Luo, Cooper, & Lindstrom, 2005; Nelson, et al., 2003) does not preclude the expression of $(\alpha4)_3(\beta2)_2$ nAChRs following chronic nicotine treatment. Both types of receptors may be present following exposure to chronic nicotine. Indeed, rubidium efflux assays have

shown that agonist-induced stimulation is biphasic with $(\alpha4)_2(\beta2)_3$ being activated at low concentrations while $(\alpha4)_3(\beta2)_2$ are activated at high concentrations following chronic nicotine exposure (Marks, Meinerz, Brown, & Collins, 2010). During withdrawal, it is possible that each type of receptor recovers from desensitization at different rates as they desensitize at different rates. Low agonist concentrations desensitize nAChRs without any apparent activation (Giniatullin, Nistri, & Yakel, 2005), which may be due to low concentrations of nicotine binding to $(\alpha4)_2(\beta2)_3$ and stabilizing the receptor in a desensitized state. Higher concentrations of nicotine might preferentially bind to a population of α4β2 nAChRs, such as the $(\alpha4)_3(\beta2)_2$, that have recovered from desensitization and left hypersensitive. Thus, the existence of two populations of α4β2 nAChRs, one that is not fully recovered from desensitization during withdrawal while the other is hypersensitive, could be one potential explanation as to why, during nicotine withdrawal, there appears to be both decreased sensitivity to nicotine at low doses and increased sensitivity to nicotine at higher doses.

Another possibility is that lower doses of nicotine are subthreshold to activate nAChRs exposed to chronic nicotine. Not only does nicotine desensitize nAChRs but it can also result in a long-term loss of function that is not due to a conformational change from the active/open state of the receptor to a desensitized state (Kuryatov, et al., 2005; Marks, et al., 1994). This long-lasting loss of nAChR function has been called functional downregulation (Marks, et al., 1993), functional inactivation (Gentry & Lukas, 2002; Lukas, 1991), and persistent inactivation (Gentry, Wilkins, & Lukas, 2003). The mechanistic differences between desensitization and a longer-lasting loss of function are unclear but it is suggested that persistent inactivation may be due to the ability of nicotine

to permeate cell membranes rather than direct effects on cell surface receptors (Gentry, et al., 2003). This suggests that there may be multiple mechanisms involved in tolerance such as receptor desensitization and longer-lasting forms of loss of nAChR function. Cell surface receptor desensitization may be a homeostatic mechanism in response to chronic nicotine that enables nAChR systems to transition towards initial levels of function even as the number of nAChRs increases due to chronic nicotine exposure. Persistent inactivation, functional inactivation, and functional downregulation may be due to the intracellular actions of nicotine. Desensitized receptors recovered during withdrawal could contribute to a hypersensitive nAChR system while longer-lasting forms of decreased function could contribute to a hyposensitive nAChR system. This could be one mechanism through which tolerance to the subjective effects of nicotine is able to persist for many years following abstinence and thus in the absence of withdrawal (Perkins, et al., 2001).

One unexpected finding from this study was that 0.022, 0.18, and 0.36 mg/kg enhanced contextual conditioning in mice withdrawn from chronic saline then tested 14 days later. Previous research has shown that that 0.18 mg/kg nicotine enhances contextual conditioning (Gould & Wehner, 1999) as well as 0.045 and 0.09 mg/kg (Gould & Higgins, 2003). In addition, 0.35 mg/kg nicotine produces a deficit in contextual fear conditioning (Gould & Wehner, 1999). In the present study, it was found that nicotine enhanced contextual conditioning across a wide range of doses (0.022, 0.09, 0.18, and 0.36 mg/kg) while 0.045 mg/kg showed a trend towards enhancement. Unlike previous research (Gould & Wehner, 1999), there were no deficits in contextual conditioning from the highest dose of nicotine tested. It is unclear why nicotine

enhanced contextual conditioning across such a broad range of doses while previous research found enhancement within a more narrow range (Gould & Higgins, 2003). One possible explanation is the amount of handling the animals received throughout the experiments. Mice in this studied underwent two surgeries prior to training in contextual conditioning, which results in more animal handling than acute nicotine studies. Previous research has shown that handling can have profound effects on behavior such as facilitating nicotine conditioned place preference in mice (Grabus, Martin, Brown, & Damaj, 2006), enhancing auditory-cue fear conditioning in rats (Hui, Hui, Roozendaal, McGaugh, & Weinberger, 2006), and reducing anxiety measured in the elevated plus maze in rats (Andrews & File, 1993). Therefore, the amount of handling the animals received in this study may have been conducive to the establishment of nicotine enhancement of contextual conditioning.

In sum, the present series of experiments provides evidence that the nAChR system is hypersensitive during nicotine withdrawal. Acute nicotine enhanced contextual conditioning across a wide range of doses, but there was a greater percent change from control in mice withdrawn from chronic nicotine for 24 hours than mice in all other withdrawal groups. The enhanced sensitivity to acute nicotine might be due to the recovery of excess nAChR function after the removal of nicotine and/or changes in nAChR stoichiometry during chronic nicotine administration. In addition, the present results suggest that there may be multiple mechanisms that contribute to tolerance such as changes in nAChR stoichiometry and the intracellular mechanisms of nicotine. Theories of nicotine addiction suggest that the recovery of nAChR function during withdrawal results in an excess excitability of the nAChR system (Dani & De Biasi, 2001; Dani &

Heinemann, 1996; Zhang, et al., 2011). This excess excitability of the nAChR system may contribute to the unrest and agitation observed in smokers during withdrawal (Dani & De Biasi, 2001), and may also alter hippocampal function resulting in cognitive deficits (Gould, et al., 2012). In addition, enhanced sensitivity to acute nicotine during a period of nicotine withdrawal might render smokers more vulnerable to forming new maladaptive drug-context associations during breaks in abstinence. There may also be enhanced sensitivity to the reinforcing and rewarding properties of nicotine during withdrawal. However, reports using self-administration indicate that the reinforcing effects of nicotine are neither enhanced nor diminished in mice undergoing spontaneous nicotine withdrawal (Semenova, Bespalov, & Markou, 2003). Other reports indicate that precipitated nicotine withdrawal decreases nicotine self-administration (Paterson & Markou, 2004). These studies suggest that there is not enhanced sensitivity to the reinforcing effects of nicotine during withdrawal. However, there may be enhanced reward sensitivity to acute nicotine during withdrawal. An interesting area of future research would be to determine if doses of acute nicotine, which are subthreshold to maintain operant responding (reinforcement) or to induce a nicotine conditioned place preference (reward), will induce a significant nicotine conditioned place preference in animals undergoing nicotine withdrawal.

CHAPTER 4

CONCLUSION

The present series of experiments examined the effects of multiple doses and durations of chronic nicotine treatment on contextual conditioning, the duration of chronic nicotine treatment required to produce nicotine withdrawal-deficits in contextual conditioning upon cessation of treatment, and the effects of acute nicotine on contextual conditioning immediately after cessation of chronic nicotine treatment and two weeks later, a time point not associated with withdrawal-deficits in learning. In Chapter 2, it was demonstrated that the ability of chronic nicotine to enhance contextual conditioning depends greatly upon the dose of nicotine as well as the duration of chronic nicotine exposure. In addition, the duration of chronic nicotine treatment required to produce tolerance was not sufficient to produce withdrawal deficits in contextual conditioning, at the same dose. This suggests that withdrawal deficits in contextual conditioning and tolerance are dissociable, operating under separate mechanisms. In addition, this suggests that withdrawal is not a simple manifestation of the same processes involved tolerance. Chapter 2 concluded with a model that integrates nAChR desensitization and upregulation to explain the present findings. Specifically, tolerance is mediated by nAChR desensitization, although there may be multiple mechanisms involved in tolerance to the enhancing and impairing effects of chronic nicotine. A combination of nAChR desensitization and upregulation contributes to doses of nicotine enhancing contextual conditioning that were previously ineffective at altering contextual conditioning. Finally, when nicotine is removed, nAChRs regain function while there are

still an upregulated number of receptors, which leads to a hyperfunctional nAChR system in the hippocampus that contributes to nicotine withdrawal-deficits in cognitive function.

The model presented in Chapter 2 suggests that the nAChR system is hyperfunctional during withdrawal. This suggestion implies that the nAChR is hypersensitive to nicotine and other nicotinic agonists during withdrawal. The experiments in Chapter 3 were designed to test the hypothesis that the nAChR system is hypersensitive during a period of nicotine withdrawal. Mice withdrawn from 12 days of chronic nicotine for 24 hours and then administered acute nicotine displayed a greater enhancement of contextual conditioning than mice in all other withdrawal groups. This finding suggests a hypersensitive nAChR system during withdrawal. However, hypersensitivity was not displayed at the lowest dose of acute nicotine. The simultaneous observation of both a hypo and hypersensitive nAChR system may be due to changes in subunit stoichiometry following chronic nicotine resulting in the expression of two populations of nAChRs that differ in sensitivity to acute nicotine. It may also be due to chronic nicotine producing long-lasting forms of reduced nAChR function that are separate from nAChR desensitization such as functional downregulation (Marks, et al., 1993), functional inactivation (Gentry & Lukas, 2002; Lukas, 1991), and persistent inactivation (Gentry, et al., 2003). The latter possibility suggests that there may be multiple mechanisms involved in tolerance. This is perhaps one reason why tolerance is observable to the effects of nicotine years following abstinence, in the absence of withdrawal-related changes and nAChR desensitization.

Tolerance and withdrawal both occur following chronic administration of nicotine; however, the results of Chapter 2 suggest that separate mechanisms underlie

each. Thus, tolerance and withdrawal might contribute differently to the development and maintenance of nicotine addiction. During the early stages of nicotine consumption, smokers might escalate their nicotine intake in order to compensate for tolerance. This escalation could lead to greater nicotine dependence and withdrawal symptoms during abstinence and suggests a role for tolerance in the early onset of dependence (Perkins 2002). However, there is very little association between tolerance and withdrawal or relapse during abstinence (Perkins et al., 2002). Rather, chronic smokers generally consume the same number of cigarettes per day on average (Jarvis, 2004) and are good at maintaining relatively constant plasma nicotine levels (Henningfield & Goldberg, 1988). These data suggest that avoidance of the symptoms associated with nicotine withdrawal, including impairments in cognitive function, plays a greater role in the maintenance of nicotine addiction than tolearnce. This supposition is supported by the finding that severity of withdrawal symptoms during abstinence predict relapse (Patterson et al., 2010). Therefore, treatments that target withdrawal symptoms may be effective at treating nicotine addiction, regardless of their effects on tolerance, and tolerance might not be a good indication of the severity of addiction or a predictor of relapse.

If treatments that target withdrawal symptoms are to be effective at treating nicotine addiction, then understanding the state of nAChRs during withdrawal is crucial to the development of these treatments. The results of Chapter 3 suggest that withdrawal from chronic nicotine, rather than prior exposure to nicotine, results in a hypersensitive nAChR in the hippocampus. This suggests that smokers who relapse during a period of nicotine withdrawal while nAChRs in the hippocampus are hypersensitive are at a greater risk for forming maladaptive drug-context associations than smokers who relapse while

the nAChR system is no longer hypersensitive. Pharmacological agents that dampen nAChR sensitivity while not inducing upregulation may then serve as effective treatments for withdrawal impairments in cognitive function during the early stages of abstinence. Sazetidine-A is a newly developed compound that has high affinity for α4β2 nAChRs that desensitizes these receptors after brief and partial agonism (Xiao et al., 2006; Zwart et al., 2008). Research with this compound has shown that it is effective at reducing nicotine self-administration in rats (Levin et al., 2010) and reversing attentional impairments produced by dizocilpine and scopolamine in rats (Rezvani et al., 2011). Therefore, sazetidine-A may also be effective at reversing nicotine withdrawal-deficits in contextual conditioning. It is unknown however whether sazetidine-A induces nAChR upregulation. Future research should be aimed at examining the cognitive effects of this compound, the effects effects of this drug on nicotine withdrawal symptoms including cognitive impairments, and the ability of this compound to induce nAChR upregulation.

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