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Identification of Pharmacological and Molecular Mechanisms involved in Nicotine Withdrawal

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INDENTIFICATION OF PHARMACOLOGICAL AND MOLECULAR MECHANISMS INVOLVED IN NICOTINE WITHDRAWAL

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

by

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"I have learned that success is to be measured not so much by the position that one has reached in life as by the obstacles which he has overcome while trying to succeed."- Booker T. Washington

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LIST OF ABBREVIATIONS

129	129P3 mice
5HT	serotonin
ACH	acetylcholine
AuIB	α-conotoxin AuIB
B6	C57Bl/6 mice
CA^{2+}	calcium
CAMKII	calcium/calmodulin-dependent protein kinase II
CPA	conditioned place aversion
CREB	cAMP response element binding protein
DA	dopamine
DHβE	dihydro-beta-erythroidine
ERK	extracellular regulated kinase
HT	heterozygote
ICSS	intracranial self stimulation
i.c.v.	intracerebroventricular
i.p.	intraperitoneal
IPN	interpeduncular nucleus
KO	knockout
LTP	long term potentiation
MII	α-conotoxin MII
MII[H9A;L15A]	α-conotoxin H9A;L15A
MEC	mecamylamine
MHB	medial habenula
MLA	methyllycaconitine
MP	mini pump
NAC	nucleus accumbens
nAChR	nicotinic acetylcholine receptor
NIC	nicotine
SAL	saline
SEM	standard error of the mean
s.c.	subcutaneous
VGCC	voltage-gated calcium channels
VTA	ventral tegmental area
WT	wildtype

Abstract

IDENTIFICATION OF PHARMACOLOGICAL AND MOLECULAR MECHANISMS

INVOVED IN NICOTINE WITHDRAWAL

By Kia Janelle Jackson, B.S.

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2008

Major Director: Dr. M. Imad Damaj
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Tobacco dependence is the leading cause of preventable death in the United States. Despite currently available smoking cessation therapies, there is a high rate of relapse in smoking among those attempting to quit. While the somatic signs of nicotine withdrawal (insomnia, increased appetite, weight gain) contribute to the continuation of smoking behavior, it has been hypothesized that the affective signs (depression, anxiety, craving, irritability) are greater motivators of relapse and continued tobacco use. There are few studies that assess the molecular and receptor-mediated mechanisms of nicotine withdrawal; therefore, our studies focus on identifying the nicotinic acetylcholine receptor

(nAChR) subtypes and post-receptor calcium-dependent mechanisms involved in nicotine withdrawal behaviors.

Using precipitated, spontaneous, and conditioned place aversion (CPA) models, we measured physical and affective signs of nicotine withdrawal in mice. Our data show that major nAChR subtypes have differential roles in nicotine withdrawal. Additionally, our results suggest a behavioral relevance for L-type calcium channels in physical nicotine withdrawal signs, while calcium/calmodulin dependent protein kinase II (CaMKII) appears to be involved in both physical and affective withdrawal behaviors.

Additionally, we conducted biochemical studies in the ventral tegmental area (VTA) and nucleus accumbens (NAc) to examine the relationship between altered withdrawal behavioral responses and calcium-dependent molecular mechanisms that contribute to nicotine withdrawal behaviors. Our results suggest an important role for β2-containing nAChRs in nicotine-withdrawal induced decreases in CaMKII and synapsin I function in the NAc.

Overall, our studies implicate a critical role for the α4α6β2* nAChR subtype in the behavioral and molecular aspects of nicotine withdrawal, thus aiding in the elucidation of nAChR subunits and mechanisms that contribute to nicotine withdrawal behaviors. The current studies are imperative for generating more successful smoking cessation therapies.

GENERAL INTRODUCTION

A. Tobacco and Nicotine Dependence

Tobacco dependence is the leading cause of preventable mortality in the United States. Maintenance of smoking behavior is largely due to nicotine, the main addictive component of tobacco (Stolerman and Jarvis, 1995; Bardo et al., 1999). Globally, smoking-related illnesses cause over four million smoking related deaths annually. Shockingly, every eight seconds, a human life is lost to tobacco use (Martin, 2002). Although many aspects of nicotine contribute to smoking, such as the rewarding and reinforcing effects, numerous studies suggest that avoidance of the negative emotional state produced by nicotine withdrawal represents a motivational component that promotes continued tobacco use and relapse after smoking cessation. In fact, the most commonly reported reason for relapsing into smoking during quit attempts is the desire to relieve the discomforts of smoking withdrawal (US Department of Health and Human Sciences, 1988). Among adult smokers, 80% report the desire to quit completely; however, those who attempt to quit on their own relapse within the first month and only 3% remain abstinent after six months (Hughes et al., 1992). While there are smoking cessation therapies available, which include nicotine replacement therapies, the anti-depressant bupropion (Zyban ®), and the partial nicotinic agonist varenicline (Chantix ®) (Cummings et al., 2006; Jorenby et al., 2006), the success rate of these therapies after one year remains only about 20-25% (Gonzales et al., 2006). Indeed, severity of the withdrawal syndrome is a better predictor of unsuccessful smoking attempts than smoke intake or dependence (West et al., 1989). Because smoking is such a widespread health

problem, it is important to understand the molecular and behavioral mechanisms of nicotine withdrawal to generate more effective smoking cessation therapies.

Smoking cessation after chronic tobacco use produces a well characterized and defined withdrawal syndrome. In humans, the nicotine withdrawal syndrome is characterized by somatic signs, which include bradycardia, gastrointestinal discomfort, and increased appetite, and affective signs, including irritability, anxiety, difficulty concentrating, disrupted cognition, nicotine cravings, depressed mood, and other mood disturbances (Stolerman and Shoaib, 1991; American Psychiatric Association, 1994; Mendreck et al., 2006). Indeed, in untreated smokers, nicotine withdrawal produces mood disturbances comparable in intensity to those seen in psychiatric outpatients (Hughes, 2006). The negative affective symptoms can start as soon as four hours after the last cigarette, peak in about three days, and are still measurable a month after cessation of tobacco use (Swan et al., 1996; Ward et al., 2001; Hughes, 2007).

Animal models of drug abuse allow researchers to investigate molecular mechanisms involved in drug abuse and potential treatments for these problems. Several groups, including our laboratory, have reported utilization of rodent models of the nicotine withdrawal syndrome. These animal behavioral models of nicotine withdrawal are discussed in more detail in a later section.

B. Molecular and Pharmacological Mechanisms of Nicotine Withdrawal

Nicotinic Receptors

A major goal in nicotine research is to gain a better understanding of the molecular and receptor mediated mechanisms of nicotine dependence and withdrawal in order to generate more effective smoking cessation therapies. The initial targets for nicotine are ligand-gated ion channels called nicotinic acetylcholine receptors (nAChRs), which have been identified throughout the central and peripheral nervous systems, as well as at skeletal neuromuscular junctions. By mimicking the effects of the endogenous transmitter acetylcholine (ACh), nicotine acts on a variety of nAChRs distributed throughout these areas, having a wide range of effects on body function, depending on the subtype composition and location. nAChRs have a pentameric structure consisting of five membrane spanning regions around a central ion channel. Muscle expresses five nAChR subunits which can coassemble to form two subtypes. These are $(\alpha1)_2\beta1\gamma\delta$ in embryonic muscle tissue, which converts to $(\alpha1)_2\beta1\varepsilon\delta$ in adult muscle tissue. Currently, twelve neuronal nAChR subunits have been identified, including α2-α10 and β2-β4, making it possible to have much larger variety in subtype composition in the brain and a much larger range of pharmacological effects in response to nicotine. The high calcium (Ca^{2+}) permeability of nAChRs in the brain and nicotine's ability to elicit neurotransmitter release by binding to neuronal nAChRs, suggests a modulatory role for nAChRs in the brain (Role and Berg, 1996). Additionally, as will be discussed in later sections, studies indicate Ca^{2+}-dependent mechanisms and nicotine-

induced neurotransmitter release as having an important role in nicotine dependence; therefore, our studies will focus on nAChRs located in the brain.

In the brain, the majority of nAChRs are presynaptically located, although some nAChR populations are found at the soma and on postsynaptic terminals (McGehee and Role, 1995; Wonnacott, 1997). Binding of a nicotinic agonist induces a change in the conformational state of the receptor. The receptor can exist in a sequence of conformational states, which are interpreted in terms of the "conformational scheme" of Katz and Thesleff (1957). These states consist of a resting state, which is inactive, yet capable of activation, an activated state where the channel opens in response to stimulation by a nicotinic agonist, and a desensitized state where the channel is inactive. Upon channel opening, there is an influx of various cations into the cell, including sodium and Ca^{2+}. With repeated exposure to nicotine, the receptor enters the desensitized state. A recent study revealed that β2* nAChR occupancy was ~88% after just one cigarette, and corresponding plasma levels were shown to lead to 50% desensitization of α4β2* nAChRs (Brody et al., 2006). Receptor desensitization after chronic nicotine exposure also leads to a compensatory upregulation of nAChRs. Indeed, postmortem binding studies have revealed a significant increase in the number of nicotine and ACh binding sites in brains of smokers compared to non-smokers (Benwell et al., 1988; Breese et al., 1997). It has been proposed that this desensitization and upregulation of nAChRs following chronic nicotine exposure is influential in producing withdrawal symptoms upon cessation of smoking (Benwell et al., 1988; Balfour and Fagerstrom, 1996; Dani and Heinemann, 1996), and that maintained nAChR

desensitization may be important for relieving nicotine withdrawal in humans (Brody et al., 2006).

Regular smokers have an excess number of nAChRs, and maintain levels of nicotine in the brain throughout the day that may keep many nAChRs in an inactive desensitized state; therefore, it has been proposed that hyperactivity of synaptic pathways due to this excess receptor number is one mechanism that may mediate withdrawal symptoms. After many hours of abstinence, such as overnight, a smoker's nicotine levels fall, returning the inactivated nAChRs to a responsive state. As a result of an excessive number of nAChRs becoming responsive, there may be abnormal enhancement of synaptic activity in non-rewarding cholinergic pathways, which may contribute to the discomfort associated with nicotine withdrawal that drives smoking behavior (Dani and Heinemann, 1996). Stabilization of nAChRs in the desensitized state with low levels of nicotine may occupy the increased pool of nAChRs and could alleviate the hyperactivity in non-rewarding cholinergic pathways (Brody et al., 2006). While this model explains mechanisms associated with spontaneous withdrawal, it does not account for symptoms that occur as a result of precipitated withdrawal. It is possible that while hyperactivity of non-rewarding pathways is one way that contributes to the manifestation of nicotine withdrawal signs, decreased activity in rewarding pathways may occur as a result of antagonist administration during chronic nicotine treatment, thus precipitating withdrawal signs.

While nicotine exerts its pharmacological and physiological effects by acting on a variety of nAChR subtypes, the main subtypes in the brain are α7* and α4β2*

(Changeux et al.,1998), where * denotes possible assembly with other nicotinic receptor subunits. The α4β2* nAChRs are thought to have the highest affinity for nicotine (McGehee and Role, 1995; Picciotto et al., 1995), while α7* nAChRs are thought to have lower affinity for nicotine (Papke and Thinschmidt, 1998). α4β2* nAChRs are found on presynaptic dopaminergic and GABAergic neurons in the ventral tegmental area (VTA), a brain area that is part of the mesocorticolimbic drug pathway which has been hypothesized to be involved in mediating both the rewarding properties of drugs, as well as the aversive behavioral state associated with nicotine withdrawal (Laviolette et al., 2002; Laviolette et al., 2008). The α5 and α6 nAChR subunits also coassemble with the β2 subunit in the mesocorticolimbic drug pathway on dopamine (DA) neurons and terminals to form functional receptors (Wada et al., 1989, 1990; Le Novère and Changeux, 1995; Le Novère et al., 1996; Klink et al., 2001). The α4α5β2 and α4α6β2 nAChR subtypes are involved in nicotine-stimulated DA release in the striatum (Champtiaux et al., 2003; Salminen et al., 2004; Lai et al., 2005). The α6 and α5 nAChR subunits are also expressed in brain areas that have been implicated as having a role in nicotine dependence behaviors. Expression of α6-containing nAChRs in the brain is largely confined to catecholaminergic nuclei, such as the VTA, substantia nigra (SN), and locus coeruleus (LC) (Le Novère et al., 1996; Klink et al., 2001), while the more widely expressed α5 nAChR subunit is found in the cerebral cortex (Gerzanich et al., 1998), cerebellum, thalamus (Flora et al.,2002) , striatum (Zoli et al.,2002), hippocampus, SN, and VTA (Wada et al., 1990), as well as peripherally in sympathetic and parasympathetic ganglia (De Biasi, 2002). The α3 subunit is coexpressed with α5

and β4 nAChRs in the peripheral ganglia, and medial habenula (MHb), and interpeduncular nucleus (IPN) (Wada et al., 1990; Zoli et al., 1995; Quick et al., 1999; Whiteaker et al., 2002). Further, α3 nAChR subunits are also expressed in brain areas which have been implicated as having a role in nicotine dependence behaviors, and can also coassemble with β2 nAChRs to form functional receptors on DA neurons and terminals (Le Novère and Changeux, 1995; Le Novère et al., 1996; Klink et al., 2001). Studies in the upcoming chapters will focus on identifying the role of these nAChR subunits in aspects of nicotine withdrawal.

Molecular Mechanisms of Nicotine Dependence and Withdrawal

The mesocorticolimbic drug pathway has been shown to be critically involved in the effects of several drugs of abuse, and is comprised of projections from the VTA to the NAc, amygdala, and prefrontal cortex. Presynaptic nAChRs are responsible for the positive modulation of neurotransmitter release (Wonnacott, 1997). Nicotine administration stimulates the release of most neurotransmitters throughout the brain, including brain regions that comprise the mesocorticolimbic drug pathway (McGehee and Role, 1995). Stimulation of neurons in the VTA via α4β2* presynaptic nAChRs leads to enhanced DA release into the nucleus accumbens (NAc). Rapid desensitization of α4β2* nAChRs on inhibitory GABAergic neurons reduces GABAergic inputs to DA neurons, thus enhancing DA transmission. Additionally, stimulation of α7* nAChRs located on excitatory glutamatergic neurons (Mansvelder and McGehee, 2002) and on DA neurons (Pidoplichko et al., 1997) can enhance glutamate release onto DA neurons, also enhancing DA transmission. This complex pattern of brain activation in response to

nicotine plays a large role in the acute rewarding and reinforcing effects of nicotine, which motivate chronic and compulsive use.

The neuroadaptations that occur as a result of chronic nicotine exposure are thought to reflect nicotine's influence on these neurotransmitter systems (De Biasi and Salas, 2008). Many studies support the vital role of the neurotransmitter DA in nicotine dependence. The nicotine induced increase in VTA DA neuron firing rate (Grenhoff et al., 1986), and subsequent DA release in the NAc is a process thought to underlie the addictive properties of nicotine (Ponteiri et al., 1996). Alternatively, studies report decreased DA neuronal activity in the VTA (Liu and Jin, 2004) and decreased DA output in the NAc after nicotine withdrawal (Hildebrand et al., 1998; Rada et al., 2001). Indeed, it is hypothesized that this relative deficiency in DA release following cessation of nicotine exposure accounts for many of the mood disorders, craving, and anhedonia that persist in many smokers long after quitting (Benowitz, 2008).

Deficits in the neurotransmitter serotonin have also been suggested to play a role in mechanisms associated with nicotine withdrawal. Clinically, the $5HT_{1A}$ receptor partial agonist buspirone shows efficacy in smoking cessation trials and may reduce severity in abstinent smokers (Hilleman et al., 1992; Schnedier et al., 1996). Serotonergic antidepressant treatment with the selective serotonin reuptake inhibitor fluoxetine and a $5HT_{1A}$ receptor antagonist was also shown to reverse the anhedonic component of nicotine withdrawal in rats (Harrison et al., 2001).

As proposed for other drugs of abuse, chronic exposure to nicotine is likely to involve modification of signaling cascades that modulate synaptic plasticity and gene

expression (Dani et al., 2001; Nestler, 2002). There are many factors by which repeated drug exposure could alter gene transcription in the brain, including altered transcription rates, altered translation of mRNA into proteins, altered transcription of mature proteins to their intracellular sites of action, and alteration of several other mechanisms involved in gene transcription and translation (Nestler et al., 2001a). Several studies have shown that chronic drug use leads to homeostatic changes in the brain that result in dependence and withdrawal symptoms (Nestler and Aghajanian, 1997); however, it has been proposed that these homeostatic changes do not explain why people relapse, even years, after cessation of drug use. An alternative hypothesis suggests that long-term associative memory processes, which receive input from midbrain DA neurons, are involved in mediating relapse after long term cessation of drug use (Berke and Hyman, 2000; Robbins and Everitt, 2002; Hyman, 2005).

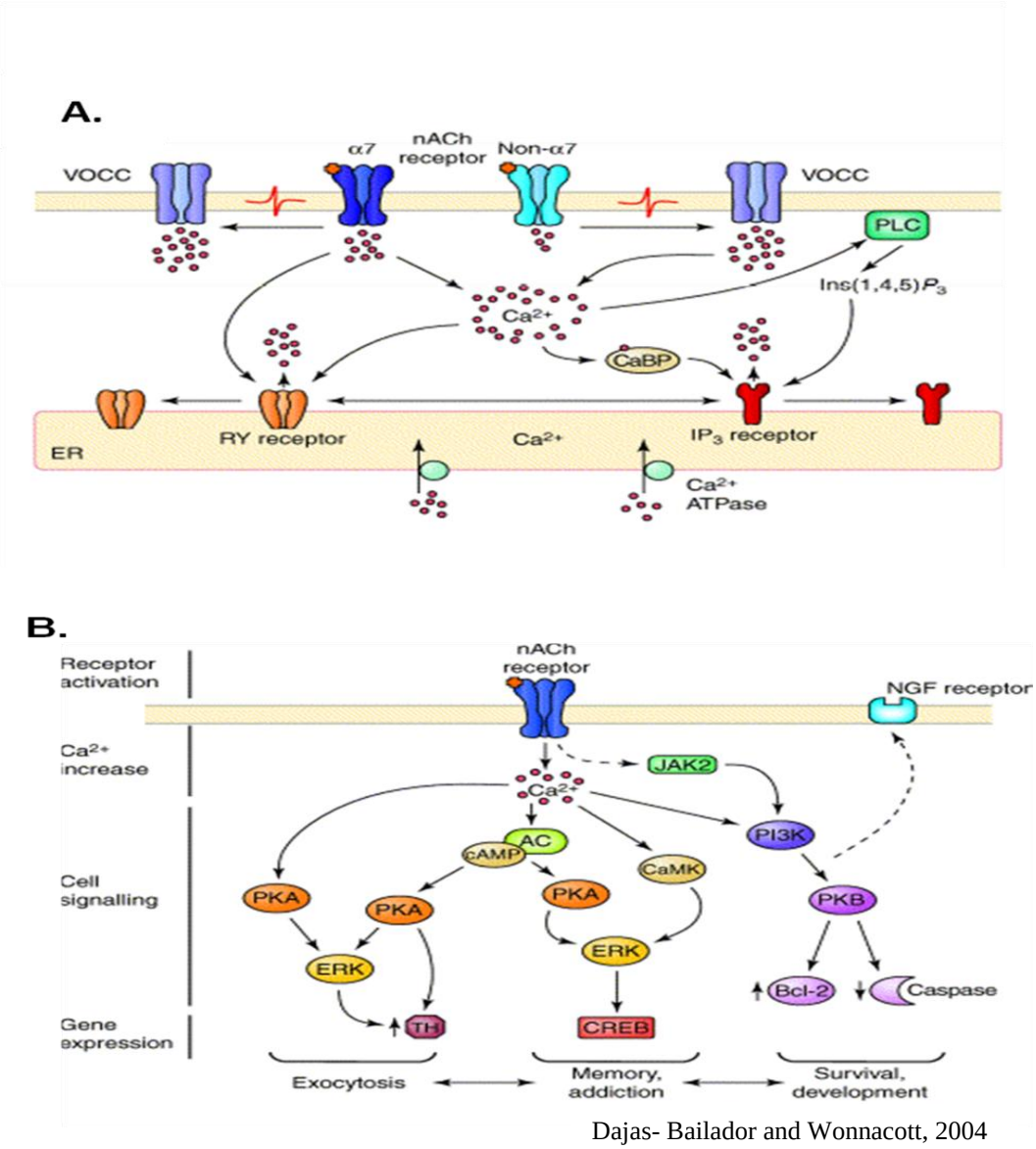
Long-term potentiation (LTP) is a mechanism characterized by a strengthening of synapses, hypothesized to play a crucial role in many forms of experience-dependent plasticity, including various forms of learning and memory (Martin et al., 2000; Malenka et al., 1989; Malenka and Bear, 2004). LTP could alter gene and protein expression in neurons, leading to reorganization of neural circuitry (Hyman et al., 2006); thus, LTP has become an important candidate mechanism involved in relapse after drug withdrawal. Evidence suggests that LTP occurs in both the VTA and the NAc (Hyman et al., 2006), and changes in some transcription factors involved in induction of LTP occur in response to drugs of abuse, including nicotine. Further, the synaptic changes that are induced by nicotine are similar to the synaptic plasticity that underlies learning

and memory (Pidoplichko et al., 2004). One transcription factor of interest is the cAMP response element-binding protein (CREB), best known for its role in learning and memory, which studies have shown to be involved in mechanisms associated with nicotine dependence, including reward (Walters et al., 2005) and withdrawal (Pandey et al.,2001; Brunzell et al., 2003; Pluzarev and Pandey, 2004). One mechanism of CREB activation is through phosphorylation by Ca^{2+}-dependent proteins. A more thorough discussion of the nicotine-induced influx of Ca^{2+}, the subsequent activation of Ca^{2+}-dependent mechanisms, and the role of Ca^{2+} in nicotine dependence and withdrawal is discussed in further detail in the next section.

C. Calcium-Dependent Mechanisms in Nicotine Dependence and Withdrawal

As a result of Ca^{2+} influx, presynaptic nAChRs facilitate Ca^{2+}-dependent release of many neurotransmitters and the activation of various downstream signaling cascades involved in gene transcription (Wonnacott, 1997). Neuronal nAChRs can elevate intracellular Ca^{2+} both directly due to the Ca^{2+} influx through the Ca^{2+} permeable nAChR channel, and indirectly because of the ability to induce membrane depolarization and activate voltage-gated Ca^{2+} channels (VGCCs), and Ca^{2+}-induced Ca^{2+} release from intracellular stores (Fig. 1A). Ca^{2+} influx through nAChRs mediates neurotransmitter release through Ca^{2+} -dependent exocytosis of synaptic vesicles and by activating Ca^{2+} -mediated cellular mechanisms. The nicotine-induced increase in intracellular Ca^{2+} initiates a cascade of second messenger events, leading to the activation of a number of Ca^{2+} -dependent targets, including Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII), CaMKIV, and protein kinase A (PKA). This leads to activation of

extracellular signal-regulated kinase or mitogen-activated protein kinase (ERK or MAPK) and CREB (Fig. 1B).



Dajas- Bailador and Wonnacott, 2004

Figure 1. Calcium-dependent mechanisms and nicotinic receptors.
A. Neuronal nAChRs can elevate intracellular Ca^{2+} both directly due to the Ca^{2+} influx through the Ca^{2+} permeable channel, and indirectly because of the ability to induce membrane depolarization and activate voltage-gated Ca^{2+} channels (VGCCs), and Ca^{2+}-induced Ca^{2+} release from intracellular stores. B. The nicotine-induced increase in intracellular Ca^{2+} initiates a cascade of second messenger events, leading to the activation of a number of Ca^{2+}-dependent targets, including Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), CaMKIV, and protein kinase A (PKA), leading to activation of extracellular signal-regulated kinase or mitogen-activated protein kinase (ERK or MAPK) and CREB.

These Ca^{2+}-dependent processes have been shown to play a role in the molecular mechanisms of the underlying synaptic plasticity involved in drugs of abuse such as cocaine (Nestler, 2001b). In fact, in the case of nicotine, decreased CREB activity was detected in the NAc (Brunzell et al., 2003; Pluzarev and Pandey, 2004), cortex, and amygdala (Pandey et al., 2001) after nicotine withdrawal, suggesting that decreased CREB activity may be associated with the dysfunction in reward mechanisms after nicotine withdrawal. Changes in CREB phosphorylation have also been observed following morphine (Lane-Ladd et al., 1997), cocaine (Kano et al., 1995) and amphetamine chronic administration (Shaw-Lutchman et al., 2003). Animals with a decrease in CREB function do not find morphine rewarding, but find cocaine more rewarding (Walters and Blendy, 2001). Taken together, CREB seems to play a major role in the cellular changes that occur in the brain after acute and chronic administration of several drugs of abuse.

There are numerous phosphorylation sites on the CREB protein that differentially regulate its activity, including a site for CaMKII. The Ca^{2+}-dependent protein, CaMKII, has not been extensively evaluated in drug abuse; however, the available studies suggest a role for CaMKII in morphine tolerance (Fan et al., 1999; Liang et al., 2004; Tang et al., 2006) and reward (Narita et al., 2004), as well as cocaine sensitization (Licata et al., 2004), and reinstatement (Anderson et al., 2008). Additionally, CaMKII has been shown *in vitro* to reduce CREB-mediated gene transcription (Matthews et al., 1994; Sun et al., 1994; Wu and McMurray, 2001). However, *in vivo,* CaMKII was shown to increase CREB activity in the spinal cord

(Miyabe and Miletic, 2005). Although the specific role is unclear, CaMKII appears to have a role in modulating CREB activity.

CaMKII is the most abundant protein kinase in the brain, comprising approximately 1-2% of total protein. Upon nicotine binding, nAChR channels open, allowing a direct influx of Ca^{2+} and other ions, as well as an indirect Ca^{2+} influx through Ca^{2+} channels. Ca^{2+} forms a complex with calmodulin, and this complex binds to and activates CaMKII. Once activated, CaMKII can auto-phosphorylate and preserve 80% of its activity, independent of Ca^{2+} (Schulman and Hanson, 1993). Presynaptically, CaMKII plays a role in neurotransmitter synthesis and release (Schulman and Hanson, 1993). Tyrosine hydroxylase, the rate-limiting enzyme in DA synthesis, and synapsin I, a vesicle protein essential for regulation of neurotransmitter release, are CaMKII substrates. Postsynaptically, CaMKII is essential for induction of LTP (Lisman et al., 2002). Due to its role in CREB modulation, LTP, and activation of other substrates involved in neurotransmitter release and gene transcription, we believe that CaMKII is a candidate protein for having a role in nicotine dependence.

The α-CaMKII mutant mouse was generated as a tool to study the role of CaMKII in LTP (Silva et al., 1992a). It was noted that mutant mice displayed some behaviors consistent with animals that have hippocampal lesions. For example, animals with hippocampal lesions exhibit increased exploratory behavior and activity when placed in an open field activity cage or Y maze (Teitelbaum and Milner, 1963; Foreman, 1983). When tested, α-CaMKII mutant mice showed increased exploratory activity in an open field and in a Y maze (Silva et al., 1992b). Additional studies using the Morris

water maze also revealed that α-CaMKII mutant mice, like hippocampal lesioned animals, have impaired spatial learning, but no deficits in contextual learning (Silva et al., 1992b). Conditional α-CaMKII mutants have also been used to study the role of α-CaMKII in learning and memory. A functional mutated form of α -CaMKII was generated *in vitro*, and a highly selective inhibitor for the mutated α -CaMKII was generated (Wang et al., 2003). Mice that overexpress the mutated α -CaMKII were generated, and it was found that the selective inhibitor could mask α -CaMKII activity in specific brain subregions of the mutated mice on the time scale of several minutes (Wang et al., 2003). Our studies using α-CaMKII mutant mice will be presented in Chapter 5.

D. Behavioral Models of Nicotine Withdrawal

Animal models of nicotine withdrawal have been developed and are useful tools for elucidating mechanisms associated with nicotine withdrawal behaviors. From these models, studies have shown that somatic signs of nicotine withdrawal are mediated by central and peripheral nAChRs, while affective signs are mediated solely through central nAChR populations (Watkins et al., 2000). Rodents are first chronically exposed to nicotine for a predetermined amount of time through osmotic mini pumps (Damaj et al., 2003; Malin et al., 2006), chronic injections (Liu et al., 2005; Miura et al. 2006), oral route via drinking water (Grabus et al., 2005; Liu et al., 2005), or intravenous administration (Wilkinson and Bevins, 2008). Withdrawal may be precipitated in chronic nicotine exposed animals with nicotinic antagonists, such as the non-selective nAChR antagonist mecamylamine, or evaluated spontaneously by the cessation of

chronic nicotine treatment. Physical signs in rodents are typically measured as somatic signs (Malin et al., 1992; Hildebrand et al., 1997; Damaj et al., 2003), hyperalgesia (Salas et al., 2004; Grabus et al., 2005), and changes in locomotor activity (Nomikos et al., 1999; Hildebrand et al., 1999), while affective signs are typically measured as anxiety-related behaviors (Damaj et al., 2003; Schneider et al., 2007; Stoker et al., 2008), elevated reward thresholds (Cryan et al., 2003; Bruijnzeel and Markou, 2004; Johnson et al., 2008), contextual fear conditioning (Davis et al., 2005), and conditioned place aversion (CPA) (Suzuki et al., 1996; Malin et al., 2006; Jackson et al., 2008).

The somatic signs of nicotine withdrawal in rodents are indicative of signs of irritation, as irritability is experienced by humans as part of the nicotine withdrawal syndrome. Hyperalgesia, or increased pain sensitivity, has also been reported in some smokers after cessation of tobacco use (Allen et al., 2000). In fact, abstinent smokers who reported having two or more withdrawal symptoms had a higher odds ratio of experiencing chronic pain in three or more locations compared to individuals who did not experience withdrawal symptoms (John et al., 2008). Changes in locomotor activity after nicotine withdrawal may reflect alterations in DA transmission. Decreases in locomotor activity, which have been reported after nicotine withdrawal in rats, correspond to a reduction in NAc and striatal DA content 24 hours after cessation of chronic nicotine infusion through osmotic mini pumps (Fung et al., 1996). Indeed, decreased DA release in the NAc has been reported after mecamylamine-precipitated withdrawal (Hildebrand et al., 1998; Carboni et al., 2000).

Mice undergoing nicotine withdrawal show increased anxiety-related behavior as measured by the plus maze (Irvine et al., 2001; Damaj et al., 2003; Jackson et al., 2008) and the light-dark box (Schneider et al., 2007; Stoker et al.,2008); thus, these models could mimic the increase in anxiety reported in humans during nicotine abstinence. Elevations in reward threshold measured by the intracranial self stimulation (ICSS) test evaluate the anhedonia, or diminished interest in rewarding stimuli, associated with nicotine withdrawal. Depression, which is highly correlated with nicotine withdrawal, also produces anhedonia (Kenny and Markou, 2001). Contextual fear conditioning explores the cognitive deficits observed after nicotine withdrawal (Davis et al., 2005), as humans report difficulty concentrating and disrupted cognition after cessation of tobacco use (Stolerman and Shoaib, 1991; American Psychiatric Association, 1994; Mendreck et al., 2006). Nicotine withdrawal also produces deficits in contextual fear conditioning in rodents, suggesting decreased cognition after nicotine withdrawal (Portugal et al., 2008). The CPA paradigm measures the overall aversion, or negative mood, associated with nicotine withdrawal (Kenny and Markou, 2001). These models have been characterized in rodents to measure the nicotine withdrawal syndrome. Current studies strive to establish more of these models in mice in order to use transgenic mice to understand the nAChRs and post-receptor mechanisms associated with nicotine withdrawal.

E. Transgenic Mice: A Complementary Approach to Pharmacological Methods

The use of genetically engineered mice, such as knockout (KO) and knockin (KI) mice, provides a powerful tool to complement results derived from pharmacological studies. Because our studies use only KO mice to examine nicotine withdrawal behaviors, this section will focus on the alternatives and limitations of the use of KO animals in behavioral studies.

The KO mouse is typically engineered using embryonic stem (ES) cells from the 129/Sv inbred mouse. The gene of interest is inactivated by replacement of coding sequences essential for gene function with a neomycin cassette. Through homologous recombination, the normal gene on the chromosome is replaced with the targeted cassette, thus "knocking out" the gene function. The ES cells from the 129/Sv mouse that contain the homologous recombination construct are injected into C57Bl/6 (B6) mouse blastocysts, and implanted into a foster mother; thus, the pups born from this event are composed of cells from a B6 embryo and 129/Sv stem cells. The chimeric mice are then mated with B6 mice to transmit the KO allele, and ultimately generate KO mice in later generations. To date, several nAChR KO mice have been generated and the phenotypes have been characterized. These include α3 (Xu et al.,1999a), α4 (Marubio et al., 1999; Ross et al., 2000), α5 (Salas et al., 2003), α6 (Champtiaux et al.,2002), α7 (Orr-Urtreger et al., 1997), α9 (Vetter et al., 1999), β2 (Picciotto et al., 1995), β3 (Booker et al., 1999), and β4 (Xu et al., 1999b).

Because of the overlapping expression patterns of nAChR subunits and the lack of selective pharmacological agents to identify specific nAChR subunits, KO mice have

proven to be useful in determining the subunit composition and physiological properties of nAChRs *in vivo*, complementing results obtained from pharmacological studies, and more recently, to test the selectivity of nicotinic receptor antibodies used for standard immunodetection procedures (Moser et al., 2007). Additionally, these genetically modified mice aid in elucidating the molecular and receptor-mediated mechanisms of many pharmacological actions of nicotine-mediated behaviors and various disease states including Alzheimer's Disease, Parkinson's Disease, and drug addiction. While it is clear that the KO approach offers many possibilities that cannot be achieved through the use of pharmacological agents, there are some important limitations to the model.

One major setback to KO studies is the occurrence of developmental modifications that may occur as a result of gene deletion, and can interfere with interpretation of results. It may be difficult to determine if the mouse phenotype is as a result of the gene deletion, or simply a mutation that reflects the importance of the gene in development. In addition, compensatory mechanisms in KO animals may raise some difficulties in data interpretation. For example, the deletion of one nAChR subunit may induce a compensatory increase in the expression of another subunit which can take over function of the deleted subunit; however, the expression level of various nAChR subunits and enzyme functions were found to be normal in various nAChR subunit KO animals (Picciotto et al., 1995; Marubio et al., 1999; Ross et al., 2000; Champtiaux et al., 2002). One minor discrepancy may be the genetic background of the mouse strain used for breeding, which has a strong influence on the expression of certain phenotypes. It was shown that the same mutation in different mouse strains can produce different

phenotypes as a result of modifier genes, or genes that affect the phenotypic expression of other genes (Nadeau, 2001). Most nAChR KO mice are backcrossed to the B6 strain; however, the use of other strains for backcrossing the mutations may result in different phenotypes. Such studies stress the importance of utilizing KO mice from the same genetic background, and of the necessity to characterize the behavioral phenotypes of the background strains prior to evaluating results using KO mice.

Our studies utilize the β2, α7, and α5 nAChR KO mice, and α-CaMKII heterozygote (HT) mice. The β2 nAChR subunit was the first subunit targeted for KO studies by Picciotto et al. (1995). *In situ* hybridization revealed normal mRNA levels of the α4, α5, β4, and β3 nAChR subunits, suggesting that these subunits were not upregulated as a result of the mutation. Studies showed that nicotine fails to stimulate the DA system in β2 KO mice (Picciotto et al., 1998); thus, several behaviors associated with nicotine-mediated DA release are abolished in β2 KO mice, including nicotine-induced locomotor activitation (King et al., 2004), nicotine discrimination (Shoaib et al., 2002), nicotine self-administration (Picciotto et al., 1998), and conditioned place preference (Walters et al., 2006), suggesting that the β2 nAChR subunit is crucial in various features associated with nicotine dependence.

Mice null for the α7 subunit used for our studies were generated as described by Orr-Urtreger et al. (1997). While the α7 subunit has broad expression in the brain, studies show that α7 KO mice show normal nicotine conditioned place preference (Walters et al., 2006), lever-pressing behavior, nicotine-induced locomotor depression, and nicotine tolerance compared to wildtpe (WT) mice (Naylor et al., 2005), suggesting

that α7* nAChRs are not involved in nicotine reward or tolerance based on these testing paradigms. The α7*nAChRs, however, are involved in nicotine withdrawal-induced hyperalgesia, as nicotine-withdrawn α7 KO do not express this response (Grabus et al., 2005). The α5 KO mouse used in our studies was generated and characterized as described by Salas et al. (2003). As with many other nAChR subunit KO mice, α5 KO mice have normal neuroanatomy and normal levels of α4, α6, α7, β2, and β4 mRNA with unchanged [^{125} I]- epibatidine and [^{125} I]α-bungarotoxin binding in the brain compared to WT littermates. While α5 KO mice were resistant to nicotine-induced seizures and to the hypolocomotive effects of nicotine (Salas et al., 2003), little work has been done to characterize the pharmacological effects of nicotine in α5 KO mice. Our experiments using these KO mice to identify nAChR subunit involvement in nicotine withdrawal are addressed in chapters 3 and 4.

F. Dissertation Objectives

The research in this dissertation focuses on identifying the major nAChR subtypes involved in nicotine withdrawal behaviors, and elucidating post-receptor Ca^{2+} dependent mechanisms that mediate withdrawal behaviors. While the positive rewarding and reinforcing effects of nicotine motivate continued use (Kenny and Markou, 2001), few studies assess the negative motivational component associated with nicotine withdrawal, although the desire to relieve the discomforts of smoking withdrawal has been reported by abstinent smokers to be the reason for relapsing into smoking during quit attempts (US Department of Health and Human Sciences, 1988). To date, the role of major nAChR subtypes in nicotine withdrawal is unclear, largely due to the low

selectivity of many nAChR agonists and antagonists. Studies addressing the signaling mechanisms involved in nicotine withdrawal behaviors are also lacking; thus, our studies aim to identify the nAChR subtypes involved in affective and somatic nicotine withdrawal behaviors, elucidate the role of Ca^{2+}-dependent mechanisms involved in nicotine withdrawal behaviors, and examine the relationship between withdrawal-induced behavioral aterations and Ca^{2+}-dependent mechanisms that mediate nicotine withdrawal behaviors.

Based on background information and our preliminary data, we hypothesized that the major nAChR subtypes have differential roles in the nicotine withdrawal syndrome. Specifically, the α4α6β2* nAChR subtype is involved in affective nicotine withdrawal behaviors. This subtype also mediates nicotine-withdrawal induced decreases in CaMKII function in the NAc. The decrease in CaMKII function leads to decreased phosphorylation of CaMKII substrates, specifically synapsin I, a protein essential for regulation of neurotransmitter release. To test our hypothesis, we began by adapting nicotine withdrawal and CPA models for mouse studies. By adapting the current nicotine withdrawal models for mouse studies, we had the advantage of testing KO mice in these models to complement pharmacological data on nicotine withdrawal studies. Using our adapted models, we addressed three specific aims.

Our first specific aim was to identify the major nAChR subtypes in affective and somatic nicotine withdrawal behaviors. These studies were conducted using pharmacological antagonists and mice null for specific nAChR subunits. We hypothesized that different nAChR subtypes have differential roles in nicotine

withdrawal. Specifically, the α4α6β2* nAChR subtype is involved in affective nicotine withdrawal behaviors, while α5*, α7*, and α3β4* nAChRs are involved in physical nicotine withdrawal.

The second specific aim was to examine the role and behavioral relevance of Ca^{2+}-dependent mechanisms, such as L-type VGCCs and CaMKII, in nicotine withdrawal. It was suggested that the sustained elevation of Ca^{2+} through nAChRs is dependent on the activation of VGCCs, largely L-type (Dajas-Bailador et al., 2002; Dickinson et al., 2007). Thus, we used L-type VGCCs selective antagonists and an agonist to elucidate the role of L-type VGCCs in affective and somatic nicotine withdrawal signs. We also chose to focus on the Ca^{2+}-dependent protein CaMKII due to its abundance in the brain and large role in mediating several proteins involved in drug-mediated behaviors. We used antagonists and CaMKII transgenic mice to examine the role of CaMKII in nicotine withdrawal. Our preliminary studies lead us to propose that LTCCs and CaMKII are relevant in nicotine withdrawal behaviors.

In the final specific aim, we investigated the relationship between the altered behavioral responses *in vivo* and the *in vitro* receptor-mediated Ca^{2+}-dependent mechanisms involved in nicotine withdrawal. Although nAChRs in the VTA are important for nicotine withdrawal behaviors, the subsequent neurotransmitter release after stimulation of VTA nAChRs occurs in the NAc, and involves presynaptic nAChRs; thus, we focused our studies on activity in the NAc after nicotine withdrawal. Using western blot analysis, we characterized the activity and expression level of CaMKII and synapsin I after acute nicotine exposure, and after nicotine withdrawal. Pharmacological

and transgenic approaches were used to determine the specific subunits through which the withdrawal-induced changes in CaMKII and synapsin I function were mediated. Our preliminary data lead is to hypothesize that CaMKII, and consequently, synapsin I activity, are decreased in the NAc after nicotine withdrawal. This decrease in activity is mediated through the α4α6β2* nAChR subtype, and is relevant to the expression of affective nicotine withdrawal behaviors.

Overall, this study identifies specific nAChR subtypes, post-receptor mechanisms, and potential brain regions important in nicotine dependence and withdrawal. Further, by showing that the decrease in CaMKII and synapsin I function, which may contribute to the decreased DA release observed after nicotine withdrawal, is mediated through the α4α6β2* subtype, which is a subtype crucial for expression of affective nicotine withdrawal signs, this study stresses the importance of affective nicotine withdrawal as a motivational component of relapse in smoking behavior. The research in this dissertation will contribute to the understanding of the molecular and receptor-mediated mechanisms of nicotine withdrawal, and provide new insight into potential targets for more effective smoking cessation therapies.

CHARACTERIZATION OF A NICOTINE CONDITIONED PLACE AVERSION MOUSE MODEL

A. Introduction

The nicotine withdrawal syndrome is characterized by both somatic and affective signs. Because of the importance of affective nicotine withdrawal signs in nicotine addiction, nicotine withdrawal studies which assess the receptor-mediated mechanisms associated with this aspect of withdrawal are currently under investigation. The relative lack of selective agonists and antagonists for the different nicotinic receptor subtypes has lead to the generation of several α-conotoxins, which have greater selectivity for specific nAChR subtypes, as well as transgenic mice, such as KO mice; thus, we have established mouse models of nicotine withdrawal in order to use transgenic mice to elucidate nicotinic receptor involvement in nicotine withdrawal.

Recently, some of the widely used affective models of nicotine withdrawal have been characterized in mice. The ICSS model, which evaluates brain reward function as a measure of the anhedonic component of withdrawal, has typically been evaluated in rats (Cryan et al, 2003; Bruijnzeel and Markou, 2004); however, recent studies have characterized the ICSS model in mice, providing the potential for measuring this affective component of withdrawal in transgenic mice (Johnson et al., 2008). Another measure of affective nicotine withdrawal used in the rat is the CPA model. The CPA model measures the aversive state associated with nicotine withdrawal. It is a form of classic Pavlovian conditioning where the animal learns to avoid a compartment that was previously paired

with an aversive stimulus. Previous work using this model in the rat has shown that nicotine withdrawal is associated with a negative affective state, and place aversion to previously neutral environmental stimuli represents a motivational component in the maintenance of drug use (Suzuki et al., 1996). The CPA protocol is advantageous over more complicated affective tests such as the ICSS protocol in that there is no need for extensive surgical procedures, and training sessions are typically shorter and less complex. Because the model also tests the animals in an antagonist-free state, it evaluates the important role of environmental stimuli in the maintenance of drug use. While the nicotine CPA model has been well defined in rats, this model has not been evaluated and characterized in mice. Indeed, the use of a mouse model would be advantageous in that it offers the possibility of exploring the underlying mechanisms of nicotine withdrawal through the use of genetically modified mice.

For our studies, because of the relative importance of affective withdrawal signs in contributing to relapse, we used two nicotine withdrawal models that would allow us to measure affective signs in mice. The plus maze model, which measures the anxiety-related component of nicotine withdrawal, will be discussed in the next chapter. The focus of this chapter is characterization of a nicotine CPA model in mice. In addition to these two models measuring different affective aspects of the nicotine withdrawal syndrome (anxiety-related behavior vs. aversion), these signs involve different brain regions, and thus, potentially different nAChR subtypes. While the dorsal raphé nucleus is crucial in mediating nicotine's effects on anxiety (Cheeta et al., 2001), the aversive response involves

the basolateral amygdala (Zanoveli et al., 2007). In the present study, we characterized a nicotine CPA model in mice to further evaluate the affective component of nicotine withdrawal. We determined the rate of acquisition, and the role of sex, age, and genotype on development of aversion. We also identified the role of specific nicotinic receptor populations through the use of various nicotinic receptor antagonists.

B. Methods

Animals

Male 129P3/J (129) and male and female B6 mice were purchased from Jackson Laboratories. The B6 mouse is an inbred strain that has been used extensively in pharmacological studies. Additionally, transgenic animals used in our studies were generated using 129 embryonic stem cells, and maintained on a B6 background. The mice were 8-10 weeks of age at the start of all studies. This is an adult age in the mouse, and published studies utilizing mice between 8-12 weeks of age do not report major behavioral differences to nicotine in mice in this age range. Male adult and adolescent ICR mice were purchased from Harlan Laboratories. Adult mice were 8-10 weeks of age and weighed approximately 20-25 g at the start of the experiment. Adolescent mice were approximately 3 weeks of age [postnatal day (PND) 21] at the start of the experiment. Animals were maintained in an Association for Assessment and Accreditation of Laboratory Animal Care-approved animal care facility and the studies were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

Drugs

(-)-Nicotine hydrogen tartrate salt, mecamylamine hydrochloride, dihydro-β-erythroidine (DHβE) and methyllycaconitine citrate (MLA) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Hexamethonium dichloride was purchased from Sigma /RBI (Natick, MA). All drugs were dissolved in physiological saline (0.9% sodium chloride) at a volume of 10 ml/kg body weight. Hexamethonium injections were administered intraperitoneally (i.p.). All other drugs were administered subcutaneously (s.c.). Doses are expressed as the free base of the drug.

Chronic nicotine administration

Mice were implanted with Alzet osmotic mini pumps [model 2002 (14 days) or model 2004 (28 days) Durect Corporation, Cupertino, CA] filled with (-)-nicotine or saline solution. The concentration of nicotine was adjusted according to animal weight and the mini pump flow rate, resulting in 36 mg/kg/day for 14 or 28 days. Because the period of early adolescence lasts approximately from PND 21 to 36, adult and adolescent mice used for the age assessment were chronically infused with 48 mg/kg/day for 7 days to ensure a sufficient level of dependence. The mini pumps were surgically implanted s.c. under sterile conditions with sodium pentobarbital anesthesia (45 mg/kg, i.p.). An incision was made in the back of the animal, and a pump was inserted. The wound was closed with wound clips, and the animal was allowed to recover on a heated pad before being returned to its home cage.

Nicotine CPA

The mice were chronically exposed to nicotine for 7 or 14 days prior to initiation of testing to induce dependence. Infusion continued throughout the duration of testing. The CPA protocol was conducted over the course of four days in a biased fashion. The CPA apparatus consisted of a three-chambered box with a white compartment, a black compartment, and a center grey compartment. The black and white compartments also had different floor textures to help the mice further differentiate between the two compartments. Day 1 of CPA testing was the pre-conditioning day. The mice were placed in the grey center compartment for a 5 minute habituation period, followed by a 15 minute test period. During habituation, mice did not have access to the other compartments. During the test period, mice were allowed to roam freely between compartments. The CPA boxes were connected to a computer, which recorded the amount of time the mouse spent in each compartment. A pre-preference score was determined for each mouse and was used to pair the mouse with the antagonist to its initially preferred compartment. On days 2 and 3 of CPA testing, all mice received injections of saline in the morning and were immediately confined to their non-drug-paired compartment for 30 minutes. No less than four hours later, mice received an injection of antagonist and were immediately confined to their drug-paired compartment for 30 minutes. Day 4 was the antagonist-free test day. Mice moved freely between compartments as on day 1 and activity counts and time spent on each side were recorded via photosensors using Med Associates interface and software. Data were expressed as time spent on drug-paired side minus time spent on saline-paired

side. A reduction in time spent in the initially preferred compartment was interpreted as CPA.

Statistical analysis

For all data, statistical analyses were performed using StatView ® (SAS, Cary, NC, USA). All studies were analyzed with one-way ANOVAs [with treatment as the between subject factor] or two-way ANOVAs [with treatment and sex as the between subject factors] using the Neuman-Keuls post-hoc test. *p* values of less than 0.05 were considered significant.

C. Results

Acquisition of aversion in the CPA model

To determine the rate of acquisition of aversion in the CPA model, mice were subjected to one, two, or three conditioning sessions with mecamylamine (3.5 mg/kg, s.c.) before the drug free test day. One mecamylamine conditioning session was sufficient to produce significant aversion in chronic nicotine infused mice. A significant aversive response was also noted after two conditioning sessions; however, there was no significant effect after three days of mecamylamine conditioning (Fig 2A). Next, it was important to determine the length of the conditioning session required to develop aversion, as well as the level of dependence necessary to precipitate an aversive response; thus, mice were placed in the chambers for 30 or 60 minutes after mecamylamine injection, and testing was initiated after 7 or 14 days of chronic nicotine infusion. Mice developed a significant CPA after 7 and 14 days of prior nicotine infusion, but only after a 30 minute mecamylamine conditioning session (Fig. 2B). No significant aversion was noted after the 60 minute conditioning session (Fig. 2B). Based on these results, for the remaining experiments, mice were chronically exposed to nicotine for 14 days prior to initiation of testing, and subjected to two 30 minute mecamylamine conditioning sessions to ensure development of aversion.

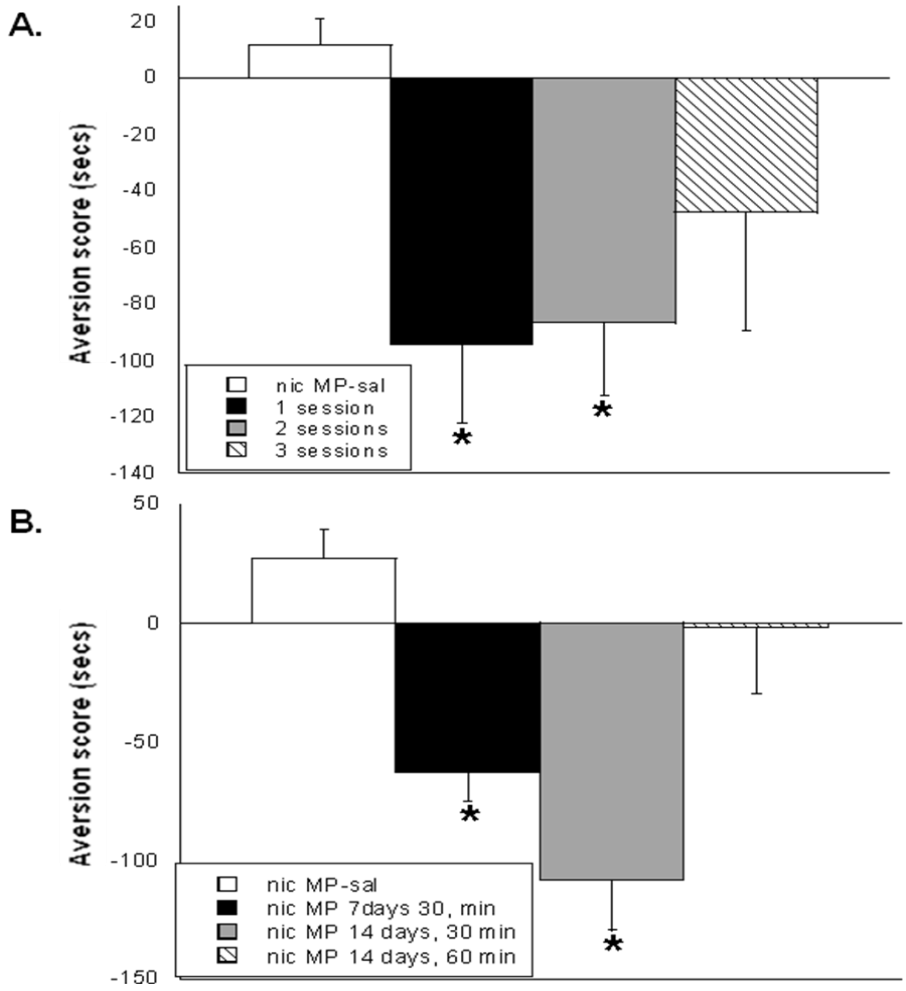


Figure 2. Acquisition of aversion in the CPA model. A. Mice acquire aversion in the CPA model after one and two conditioning sessions; however, no significant effect was observed after three conditioning sessions. B. Aversion is precipitated in mice chronically infused with nicotine for 7 and 14 days prior to test initiation. 30 minute conditioning sessions are sufficient to develop aversion. The effect is lost after 60 minute conditioning sessions. Each point represents ± S.E.M. of 10-12 mice per group. * denotes $p<0.05$.

Assessment of various antagonists in the CPA model

The role of various nicotinic receptors in the aversion associated with nicotine withdrawal was evaluated using nicotinic receptor antagonists. We first wanted to determine the involvement of central and peripheral nAChR populations in nicotine- withdrawal aversion. Mecamylamine, a non-selective receptor antagonist that blocks both central and peripheral nicotinic receptors, dose-dependently precipitated aversion in mice. A significant CPA was observed with 3.5 mg/kg (s.c.) mecamylamine, but not with 1 or 2 mg/kg (Fig. 3A). Further, the highest dose of mecamylamine used for the studies did not precipitate aversion in chronic saline infused mice, suggesting that the dose used is not behaviorally active by itself (Fig. 3B). Hexamethonium (1 and 5 mg/kg, i.p.), a peripheral nicotinic receptor antagonist that does not cross the blood brain barrier, did not produce significant aversion as seen in mice treated with mecamylamine (Fig. 3C). Because the α4β2 and α7 subtypes are major subtypes in the brain, we wanted to evaluate the role of these receptor subtypes in nicotine withdrawal. We measured aversion after conditioning with the β2-selective antagonist, DHβE, and the α7 antagonist, MLA. DHβE dose-dependently precipitated aversion at 0.5 and 2 mg/kg (s.c.), while MLA did not precipitate aversion at 10 mg/kg (s.c.), a dose that effectively blocks α7 nicotinic receptors (Ward et al., 1990) (Fig. 3D).

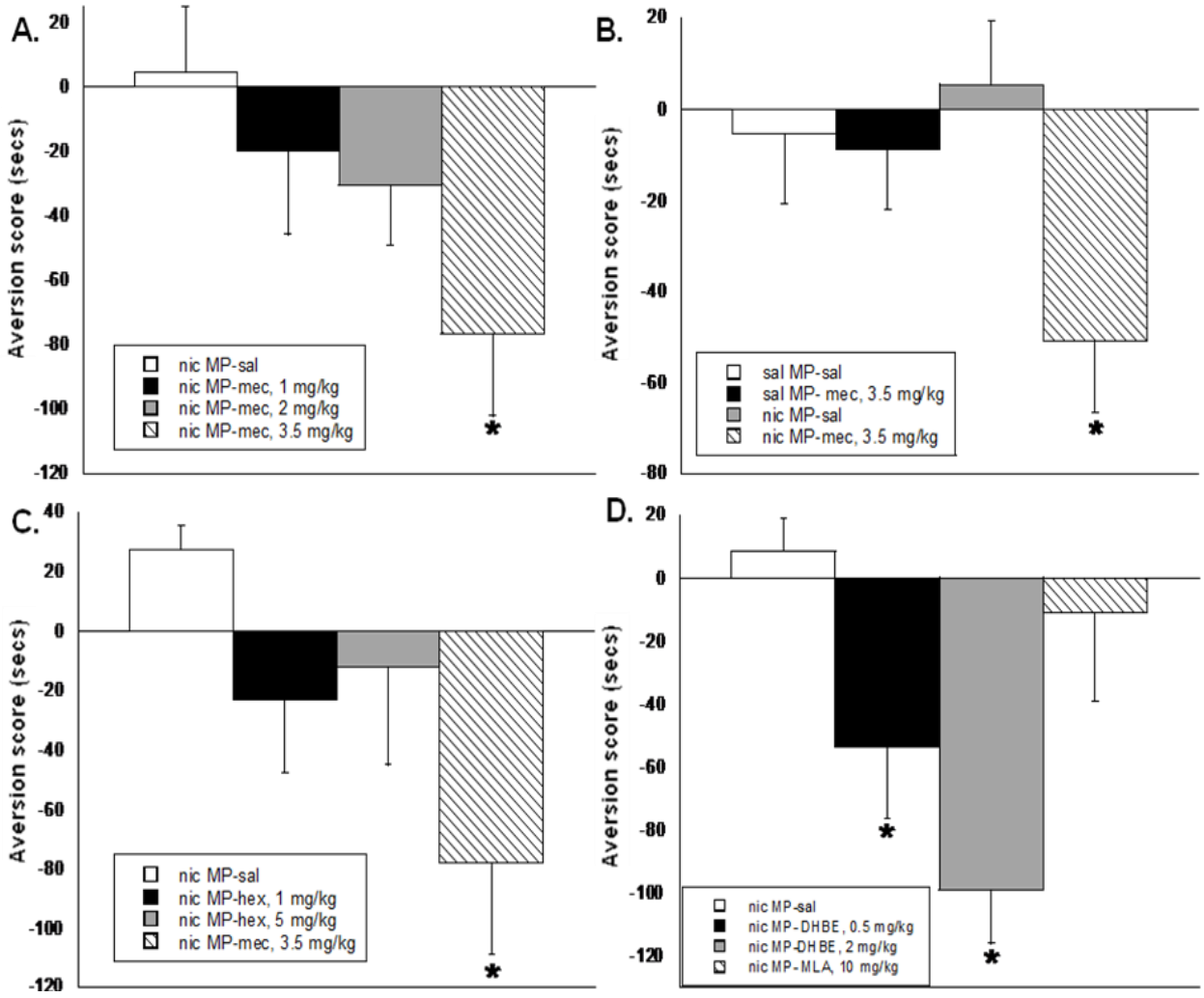


Figure 3. Assessment of various antagonists in the CPA model. A. Mecamylamine dose-dependently precipitates aversion in the CPA model. B. The dose of mecamylamine used in the model, 3.5 mg/kg, does not precipitate aversion in saline infused mice, suggesting that the dose is not behaviorally active by itself. C. Hexamethonium (hex), a peripheral nicotinic receptor antagonist, does not precipitate aversion in chronic nicotine infused mice. D. The β2-selective antagonist, DHBE, but not the α7 antagonist, MLA, dose-dependently precipitates aversion in chronic nicotine infused mice. Each point represents ± S.E.M. of 10-12 mice per group. * denotes $p<0.05$ vs. saline groups.

Sex and strain assessment in the CPA model

The transgenic mice used in our studies are maintained on a B6 background and derived from 129 embryonic stem cells. It was therefore necessary to evaluate the background strains of our transgenic mice in order to make the most efficient interpretation of our KO results. Additionally, because of the limited number of KO animals produced in each litter, it is sometimes necessary to use both male and female animals for our studies; thus, we also evaluated differences between male and female B6 mice in our CPA model. Results show that mecamylamine (3.5 mg/kg, s.c.) precipitated aversion in male B6 and 129 mice (Fig. 4). While there was a trend toward a more severe aversive response in B6 mice than 129 mice, the difference was not statistically significant ($p = .25$ for B6 vs. 129 mice) (Fig.4). Female B6 mice were also evaluated in the nicotine CPA model. As noted, mecamylamine (3.5mg/kg, s.c.) precipitated significant aversion in male B6 mice, but CPA was absent in female B6 mice (Fig. 5). Based on these results, only male mice were used for subsequent studies.

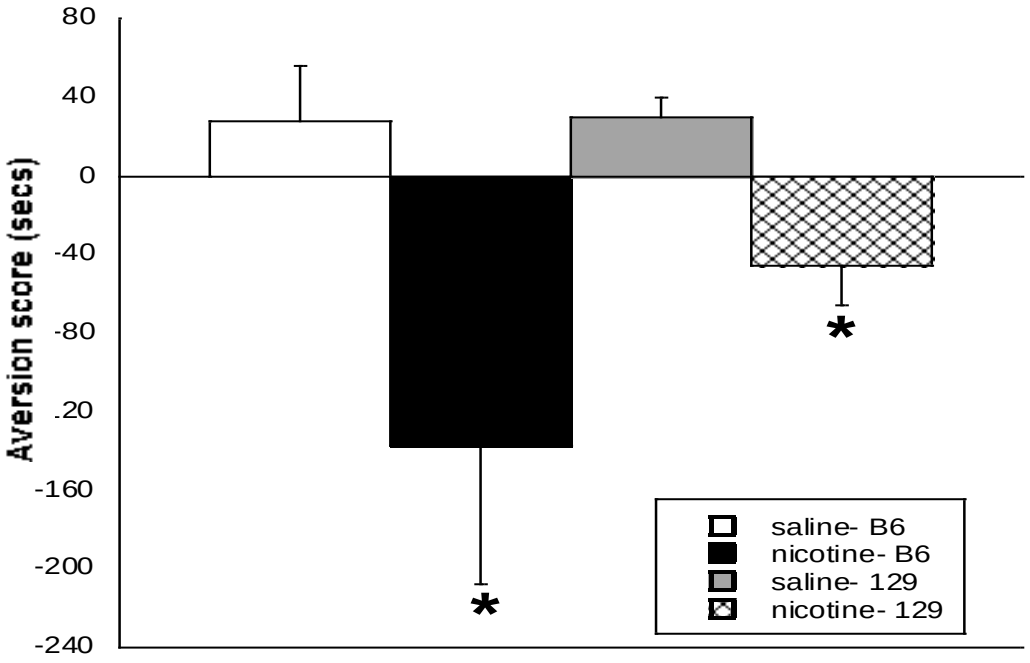


Figure 4. CPA assessment using 129 and B6 male mice. Mecamylamine precipitates aversion in the 129 and B6 inbred strains. Although there was a trend toward a higher level of aversion in B6 mice, the difference was not significant (p= .25 for B6 vs. 129 group). Each point represents ± S.E.M. of 12 mice per group. * denotes p < 0.05 vs. the corresponding saline group.

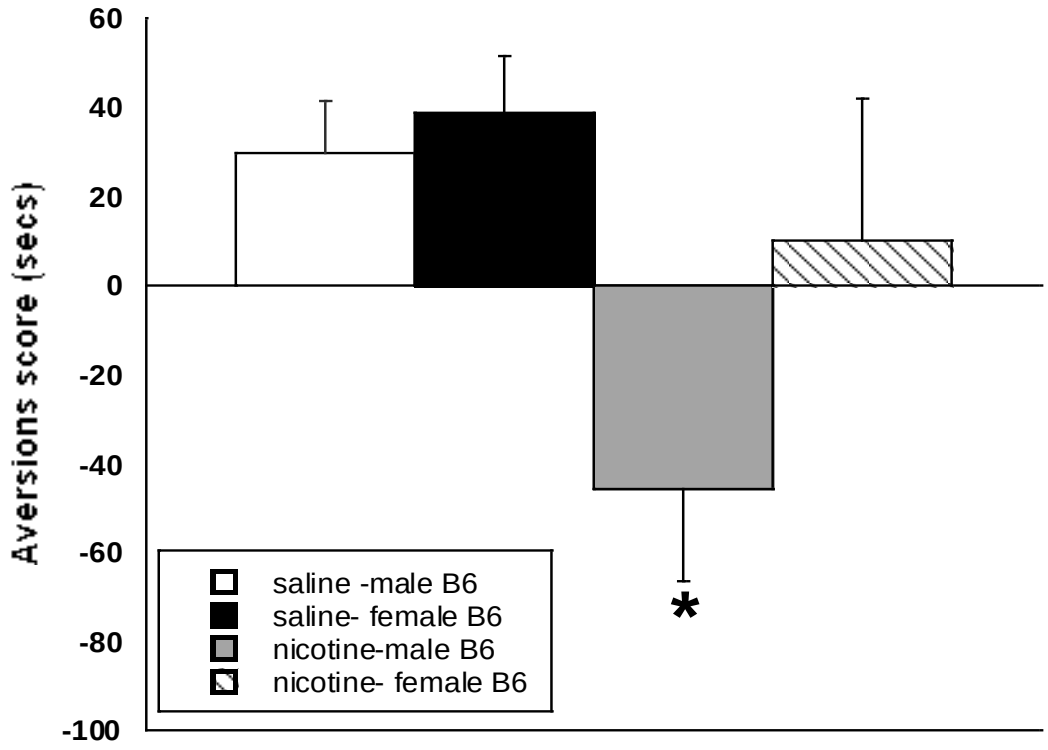


Figure 5. Assessment of male and female B6 mice in the development of aversion in the CPA model. Chronic nicotine infused male B6 mice develop aversion in the CPA model; however, nicotine infused female B6 mice do not develop aversion in this testing scheme. Each point represents ± S.E.M. of 12 mice per group. * denotes $p<0.05$ vs. the corresponding saline group.

Evaluation of age differences in the CPA model

To complete characterization of a nicotine CPA model in mice, we wanted to evaluate the importance of age in the development of CPA. Early adolescent and adult mice were tested in the CPA model to evaluate age effects. Mecamylamine (3.5 mg/kg, s.c.) precipitated significant aversion in adult mice, but no significant effect was observed in adolescent mice, suggesting that adolescents are less sensitive to the aversive effects of nicotine withdrawal (Fig. 6). By itself, mecamylamine did not precipitate significant aversion in adult or adolescent saline controls, suggesting that the dose used was not behaviorally active in saline mice.

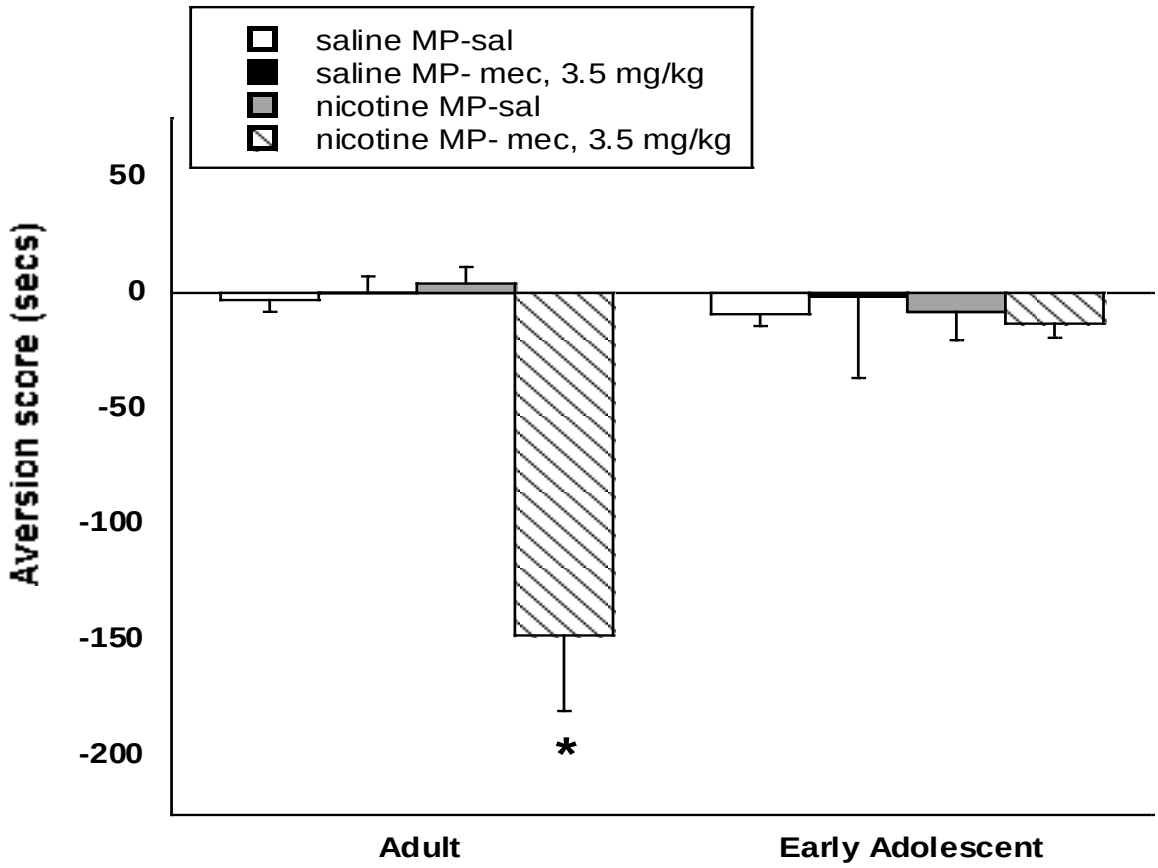


Figure 6. Age differences in development of aversion in the CPA model. A. Mecamylamine (3.5 mg/kg, s.c.) precipitates aversion in adult mice, but B. not in early adolescent mice. The dose of mecamylamine used did not precipitate aversion in saline-treated adult or adolescent mice. Each point represents ± S.E.M. of 10-12 mice per group. * denotes $p<0.05$ vs. control groups.

D. Discussion

The present research aimed to characterize a nicotine CPA model in the mouse. The adaptation of such a model for mouse studies allows us to define the underlying receptor mechanisms of affective nicotine withdrawal through the use of genetically modified mice. The major findings showed that mecamylamine and DHβE, but not hexamethonium or MLA, precipitated significant aversion in the CPA model, suggesting that the aversion associated with nicotine withdrawal is mediated by central populations of β2-containing nAChRs. Further, we show that sex and age are contributing factors to the development of nicotine CPA.

Acquisition of a significant CPA was observed after one and two conditioning sessions, but surprisingly, a significant effect was lost after three conditioning sessions. It is possible that some adaptation to the aversive stimuli occurred during the three day consecutive conditioning, thus decreasing the degree of CPA expression on test day. In the current study, mice were conditioned to mecamylamine for three consecutive days. Prior nicotine CPA studies conducted in rats used one (Suzuki et al., 1996; Suzuki et al., 1999; Ise et al., 2002; Malin et al., 2006) or four mecamylamine conditioning sessions on alternating days (O'Dell et al., 2007). In another recent study by Guillem et al. (2007), rats were treated for five consecutive conditioning sessions with no adaptation to the aversive stimuli; however, this study was conducted in rats, where species differences could be a factor. Conditioning sessions in the Guillem study were also shorter than our sessions, and were initiated 3 days after mini pump implantation; hence, differences in the level of

nicotine dependence are possible. Results also showed that 7 or 14 days of prior nicotine exposure was sufficient to obtain mecamylamine-precipitated aversion after a 30 minute, but not 60 minute conditioning session. The half-life of mecamylamine in the rodent is approximately 1 hour (Debruyne et al., 2003); therefore, it is possible that mecamylamine's effects in the mouse began to diminish during the 60 minute conditioning session. In our study, a fairly high dose of mecamylamine (3.5 mg/kg) was necessary to precipitate a significant CPA. Significant aversion was not precipitated at 2 mg/kg mecamylamine; thus, it is possible that the half-life dose of mecamylamine is not sufficient to precipitate significant CPA in chronic nicotine infused mice.

Results using different antagonists suggest the involvement of central, but not peripheral nicotinic receptor populations. While mecamylamine, a non-selective nicotinic receptor antagonist that acts on both central and peripheral nicotinic receptor populations, precipitated significant aversion in chronic nicotine infused mice, hexamethonium, a peripheral nicotinic receptor antagonist that does not cross the blood brain barrier, did not precipitate aversion at any dose tested. These results are consistent with studies which indicate that affective nicotine withdrawal signs are mediated solely by central nicotinic receptor populations (Watkins et al., 2000). Additionally, results suggest that the β2, but not the α7 nicotinic receptor subunit, is involved in development of nicotine CPA. DHβE, a β2-selective antagonist, dose-dependently precipitated a significant CPA, while MLA did not precipitate CPA at a dose that effectively blocks α7 nicotinic receptors (Ward et al., 1990). The current results are consistent with previous studies from our lab showing that

DHβE precipitates anxiety-related behavior in the plus maze test in nicotine-dependent mice (Damaj et al., 2003). It is also noted that DHβE was more potent than mecamylamine in this assessment, as DHβE was able to precipitate aversion at lower doses than mecamylamine. Taken together, the results suggest that β2-containing nicotinic receptor subtypes are involved in affective nicotine withdrawal behaviors and that CPA is a centrally mediated effect. To further evaluate these conclusions, a complementary approach using nAChR KO mice to evaluate nAChR subunit involvement in the CPA model is presented in chapters 3 and 4.

Our evaluation using male and female B6 mice suggests that sex factors contribute to the development of nicotine withdrawal aversion. Mecamylamine precipitated a significant CPA in male, but not female B6 mice. Prior evaluations of female mice in nicotine withdrawal revealed that female mice did not express an anxiety-related response, suggesting that female mice are less sensitive to affective nicotine withdrawal (Kota et al., 2008). Another explanation could be the influence of sex hormones. Indeed, sex hormones can modulate the effects of nicotine and may contribute to differences in nicotine's responses between males and females (Damaj, 2001). Human studies also suggest that hormonal changes during different menstrual cycle phases impact severity of the nicotine withdrawal syndrome (Carpenter et al., 2006). In the current study, we did not control for the estrous cycle in female mice. Consequently, it is possible that the female mice were in different phases of the estrous cycle, thus impacting our results. Results also suggest that genotypic factors may contribute to development of nicotine CPA. While significant

aversion was precipitated in both male 129 and B6 mice, aversion appeared to more intense in B6 mice, although the difference in aversion scores was not significant. These results are consistent with results showing that physical nicotine withdrawal and anxiety-related behavior is more intense in B6 mice than 129 mice (Damaj et al., 2003). Studies testing additional mouse strains may be necessary to support a role for the involvement of genotypic factors in the development of nicotine CPA.

In our final assessment, results revealed a significant contribution of age to the development of significant aversion. Mecamylamine (3.5 mg/kg, s.c.) precipitated aversion in chronic nicotine infused adult, but not adolescent mice. Our findings are consistent with previous findings from Kota el al. (2007), showing that male adolescent mice are less sensitive to the affective measures of nicotine withdrawal than male adult mice, as adolescents do not express an anxiety-related response on the plus maze. Further, O'Dell et al. (2007) showed that CPA was lower in chronic nicotine infused adolescent rats versus adult rats using a biased experimental design. It is possible that adolescent rodents may be less able than adults to associate environmental cues with aversive nicotine withdrawal effects; however, the aforementioned study addressed this possibility using another aversive stimulus, lithium chloride (LiCl) injections. The effect was shown to be specific to nicotine withdrawal aversion, as there was no significant difference between adolescents and adults in learning place aversion to LiCl (O'Dell et al., 2007). Additionally, in the current study, two conditioning sessions were used to acquire a nicotine CPA. The possibility that adolescent mice require more conditioning sessions to acquire a significant

CPA cannot be ruled out; however, such an assessment was beyond the scope of the current study.

In summary, the results of our study demonstrate that our mouse nicotine withdrawal models are useful in assessing the pharmacological, biochemical, and genetic mechanisms associated with a motivational component of drug dependence. Further, due to sex and age differences that may confound our results, only male adult KO mice were used in our nicotine withdrawal assessments. The next several chapters will utilize the CPA model, as well as precipitated and spontaneous nicotine withdrawal models, to characterize nAChR involvement and post-receptor mechanisms that contribute to nicotine withdrawal behaviors.

THE ROLE OF α4α6β2* NICOTINIC RECEPTORS IN NICOTINE WITHDRAWAL

A. Introduction

The β2 nAChR subunit is a central, highly expressed subunit that coassembles with many subunits to form functional receptors, including α4, α5, and α6 nAChRs, in the mesocorticolimbic drug pathway on DA neurons and terminals (Wada et al., 1989, 1990; Le Novère and Changeux, 1995; Le Novère et al., 1996; Klink et al., 2001). The α4α5β2* and α4α6β2* nAChR subtypes are involved in nicotine-stimulated DA release in the striatum (Champtiaux et al., 2003; Salminen et al., 2004; Lai et al., 2005). The α6 and α5 nAChR subunits are also expressed in brain areas that have been implicated as having a role in nicotine dependence behaviors. Expression of α6-containing nAChRs in the brain is largely confined to catecholaminergic nuclei, such as the VTA, SN, and LC (Le Novère et al., 1996; Klink et al., 2001), while the more widely expressed α5 nAChR subunit is found in the cerebral cortex (Gerzanich et al., 1998), cerebellum, thalamus (Flora et al.,2002) , striatum (Zoli et al.,2002), hippocampus, SN, and VTA (Wada et al., 1990), as well as peripherally in sympathetic and parasympathetic ganglia (De Biasi, 2002).

Studies have utilized the available nicotine withdrawal models to assess nAChR involvement in nicotine dependence behaviors, both pharmacologically, and using transgenic animals. The β2-selective antagonist DHβE was shown to precipitate anxiety-related behavior (Damaj et al., 2003) and elevations in reward threshold in chronic nicotine

infused rodents (Bruijnzeel and Markou, 2004), implicating a role for β2-containing nAChRs in affective withdrawal behaviors. The α6 subunit plays a role in the locomotor stimulating effects of nicotine (Le Novère et al., 1999), an effect suggested to be due to enhanced mesolimbic DA transmission (Benwell and Balfour, 1992). Furthermore, studies show that α5 KO mice are resistant to nicotine-induced seizures (Salas et al., 2003). While the available studies provide some insight into the nAChR subtypes involved in nicotine withdrawal, and suggest a possible role for these subunits in nicotine addictive behaviors, evidence of a behavioral role for β2, α5, and α6 nAChRs is still lacking. Despite the importance of affective signs in contributing to relapse, few studies address this aspect of withdrawal. Additionally, many studies utilize nAChR antagonists, which do not have high selectivity for specific subunits. A complementary approach would be the use of transgenic mice for specific nicotinic receptor subunits. Indeed, the use of nAChR KO mice provides greater specificity than would be achieved using current pharmacological agents.

In the current study, using precipitated, spontaneous, and CPA models of nicotine withdrawal, we determined the contribution of the β2, α6, and α5 nAChR subunits in both physical and affective signs of nicotine withdrawal using β2 and α5 nAChR KO mice and the α6-selective nAChR antagonist α-conotoxin H9A;L15A (MII[H9A;L15A]) (McIntosh et al., 2004).

B. Methods

Animals

Male B6 mice were obtained from Jackson Laboratories. Breeding pairs of mice lacking the β2 subunit of the nicotinic receptor (B6 background) and WT littermates were shipped from Institut Pasteur, Paris, France (see Picciotto et al., 1995 for information regarding initial breeders). Mice null for the α5 nicotinic receptor subunit (B6 background) and WT littermates were shipped from Baylor College of Medicine, Houston, Texas (see Salas et al., 2003 for information regarding initial breeders). For all experiments, α5 KO mice were backcrossed to at least 8-10 generations, and β2 KO mice were backcrossed at least 10-12 generations. Mutant and WT controls were obtained from crossing HT mice. This breeding scheme controlled for any irregularities that might occur with crossing solely mutant animals.

Drugs

(-)-Nicotine hydrogen tartrate salt and mecamylamine hydrochloride were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Drugs were dissolved in physiological saline (0.9% sodium chloride) and injected s.c. at a volume of 10 ml/kg body weight. All doses are expressed as the free base of the drug. The α6*-selective antagonist, MII[H9A;L15A], was synthesized as previously described in McIntosh et al. (2004). The highest dose for MII[H9A;L15A] (30 pmol) intracerebroventricular (i.c.v.) injection was calculated based on the functional IC_{50} at the α6 subunit (McIntosh et al., 2004).

Chronic nicotine administration

Mice were implanted with Alzet osmotic mini pumps [model 2002 (14 days) or model 2004 (28 days) Durect Corporation, Cupertino, CA] filled with saline or (-)-nicotine (36 mg/kd/day) as described in Chapter 2.

I.c.v. surgery

Mice were anesthetized with sodium pentobarbital (45 mg/kg, i.p.) on the evening prior to testing, and a scalp incision was made to expose the bregma. Unilateral injection sites were prepared using a 26-gauge needle with a sleeve of polyurethane (PE) tubing to control depth of the needle at a site 2 mm rostral and 2 mm lateral to the bregma at a depth of 2 mm. Animals were sutured in such a way to enable an injection volume of 5 µl using a 26-gauge needle with a sleeve of PE tubing into the lateral ventricle on the morning of testing. The needle was held in place for 20 seconds to ensure drug delivery.

Nicotine withdrawal assessment

Mice were implanted with mini pumps containing nicotine or saline for 14 days. On the morning of day 15, mice were injected with mecamylamine (2 mg/kg, s.c.) or saline, and withdrawal signs were measured 10 minutes after injection. The mice were first evaluated for 5 minutes in the plus maze test for anxiety-related behavior. The mice were then observed for somatic signs of withdrawal for 20 minutes. Hyperalgesia was evaluated immediately after the somatic sign observation period. The specific testing sequence was chosen based on prior studies from the lab showing that this order of testing reduced within-group variability and produced the most consistent results. For spontaneous

withdrawal studies, mini pumps were removed on the morning of day 14 and testing was initiated 18-24 hours later, on day 15.

Elevated plus maze. An elevated plus-maze, prepared with grey Plexiglas, consisted of two open arms (23 x 6.0 cm) and two enclosed arms (23 x 6 x 15 cm in wall height) that extended from a central platform (5.5 x 5.5 cm). It was mounted on a base raised 60 cm above the floor. Fluorescent lights (350 lux intensity) located in the ceiling of the room provided the only source of light to the apparatus. The animals were placed in the center of the maze, and allowed to roam freely between the open and closed arms. The time spent in the open and closed arms was automatically recorded by a photocell beam system. The test lasted 5 minutes, and the apparatus was thoroughly cleaned after removal of each animal. A decrease in the amount of time spent on the open arms was indicative of increased anxiety-related behavior. Results were expressed as the mean ± S.E.M. number of seconds spent in the open arms. As a control, the number of times each animal crossed from one side of the plus maze to the other was measured, noted as the total average number of arm crosses. This was to ensure that the reduction in time spent on the open or closed arms was not a reflection of a lack of overall activity.

Somatic signs. Mice were observed for 20 minutes in empty transparent activity cages (32 x 18 cm) for typical somatic withdrawal behaviors. Typical nicotine withdrawal signs that were tallied included head shakes, paw tremors, body tremors, and backing. Ptosis, curls, and jumps were also tallied collectively as "other" somatic signs. Results were expressed

as the mean ± S.E.M. number of signs displayed by mice during the 20 minute observation period.

Hyperalgesia. The nicotine withdrawal-induced hyperalgesia response was evaluated using the hot plate test (Thermojust Apparatus). The hot plate is a rectangular heated surface surrounded by plexiglass and maintained at 52°C. The device is connected to a manually operated timer that records the amount of time the mouse spends on the heated surface before showing signs of nociception (e.g. jumping, paw licks). The timer has an automatic cut-off of 40 seconds to avoid tissue damage. A decreased latency on the hot plate was counted as increased pain sensitivity (hyperalgesia). Results were expressed as the mean ± S.E.M. latency (reaction time for jumping or paw-licking) displayed by the mice.

Nicotine CPA

β2 and α5 assessment

Mecamylamine-precipitated CPA for β2 and α5 KO mice was conducted using male KO and WT littermates as described in Chapter 2.

α6 assessment

After the mecamylamine conditioning session on the evening of day 3, i.c.v. injection sites were prepared. On day 4, mice received i.c.v. injections of vehicle or MII[H9A;L15A] (22.5 or 30 pmol), 5 minutes before being placed in the test chambers.

Statistical analysis

For all data, statistical analyses were performed using StatView ® (SAS, Cary, NC, USA). Studies using transgenic mice were analyzed with two-way ANOVAs [with genotype and treatment as between subject factors] using the Neuman-Keuls post-hoc test. *p* values of less than 0.05 were considered significant.

C. Results

Evaluation of affective and physical signs of nicotine withdrawal using the precipitated model

To assess the involvement of specific nAChR subtypes in nicotine withdrawal, a nicotine withdrawal model was adapted to allow measurement of both physical and affective aspects of nicotine withdrawal in one setting. Mecamylamine (2 mg/kg, s.c.) precipitated significant nicotine withdrawal signs in mice chronically exposed to nicotine (36 mg/kg/day) for 14 days (Fig. 7). Results show a significant reduction in the amount of time spent on the open arms of the elevated plus maze in chronic nicotine infused mice treated with mecamylamine when compared to control groups, indicating an anxiety-related response in these mice (Fig. 7A). The average number of arm crosses was tallied as a measure of locomotor activity on the plus maze. No significant difference in the number of crosses between arms was noted between groups, suggesting that the decrease in open arm time was not attributed to reduced activity on the plus maze (Table 1). Nicotine-dependent mice also showed enhanced nicotine withdrawal somatic signs after mecamylamine injection (Fig. 7B), as well as significant nicotine-withdrawal induced hyperalgesia as measured by a decreased latency on the hot plate compared to saline controls (Fig. 7C). Saline mini pump mice that received an injection of mecamylamine and nicotine mini pump mice that received an injection of saline on test day did not differ from saline-saline control animals, indicating that the mecamylamine dose utilized did not produce effects on its own, and that the nicotine mini pump mice were not experiencing nicotine withdrawal on test day.

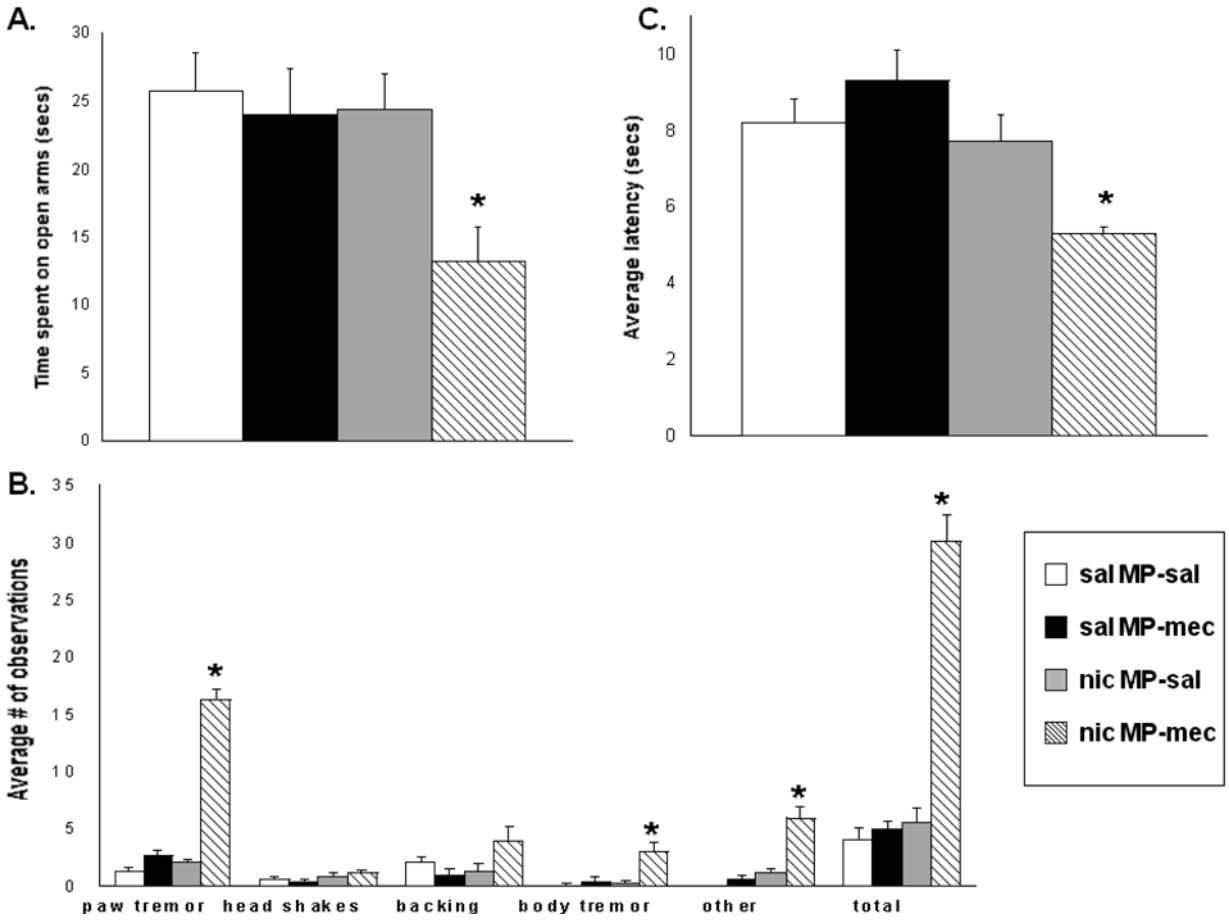


Figure 7. Assessment of physical and affective nicotine withdrawal signs in B6 mice using the precipitated model. After treatment with mecamylamine (2 mg/kg, s.c.) on test day, nicotine-dependent mice show A. anxiety-related behavior noted by a reduction in the time spent on the open arms, B. significant somatic signs, and C. withdrawal-induced hyperalgesia, noted by a decreased latency on the hotplate. Each point represents the mean ± S.E.M. of 8 mice per group. * denotes $p < 0.05$ vs. control groups.

Table 1: Total average number of arm crosses in the plus maze after precipitated nicotine withdrawal.

Using the precipitated model, nicotine-dependent mice were treated with mecamylamine (2 mg/kg, s.c.) on test day (day 15), and the total number of crosses between open and closed arms of the plus-maze test was counted. Numbers are presented as the total average number of arm crosses ± SEM for 8 mice per group.

B6 mice	Sal-Sal	Sal-Mec	Nicotine-Sal	Nicotine-Mec
	3.7 ± 0.64	4.2 ± 0.67	3.3 ± 0.42	4.2 ± 0.51

Role of the β2 nAChR subunit in nicotine withdrawal

The 14 day – precipitated nicotine withdrawal model was used to measure the affective and physical signs of nicotine withdrawal in chronic nicotine infused β2 KO mice. Results in Figure 8A show that chronic nicotine infused β2 KO mice displayed a loss of withdrawal induced anxiety-related behavior when compared to WT counterparts, indicated by no difference in the amount of time spent on the open arms of the place maze compared to control animals. No significant difference in the number of arm crosses between groups was noted (Table 2). However, chronic nicotine infused β2 KO and WT mice both displayed significantly more somatic signs than saline infused WT and KO mice (Fig. 8B) and displayed a similar decrease in hot plate latency, indicating a significant hyperalgesia response (Fig. 8C). Saline control β2 KO mice did not differ from WT counterparts in any withdrawal test.

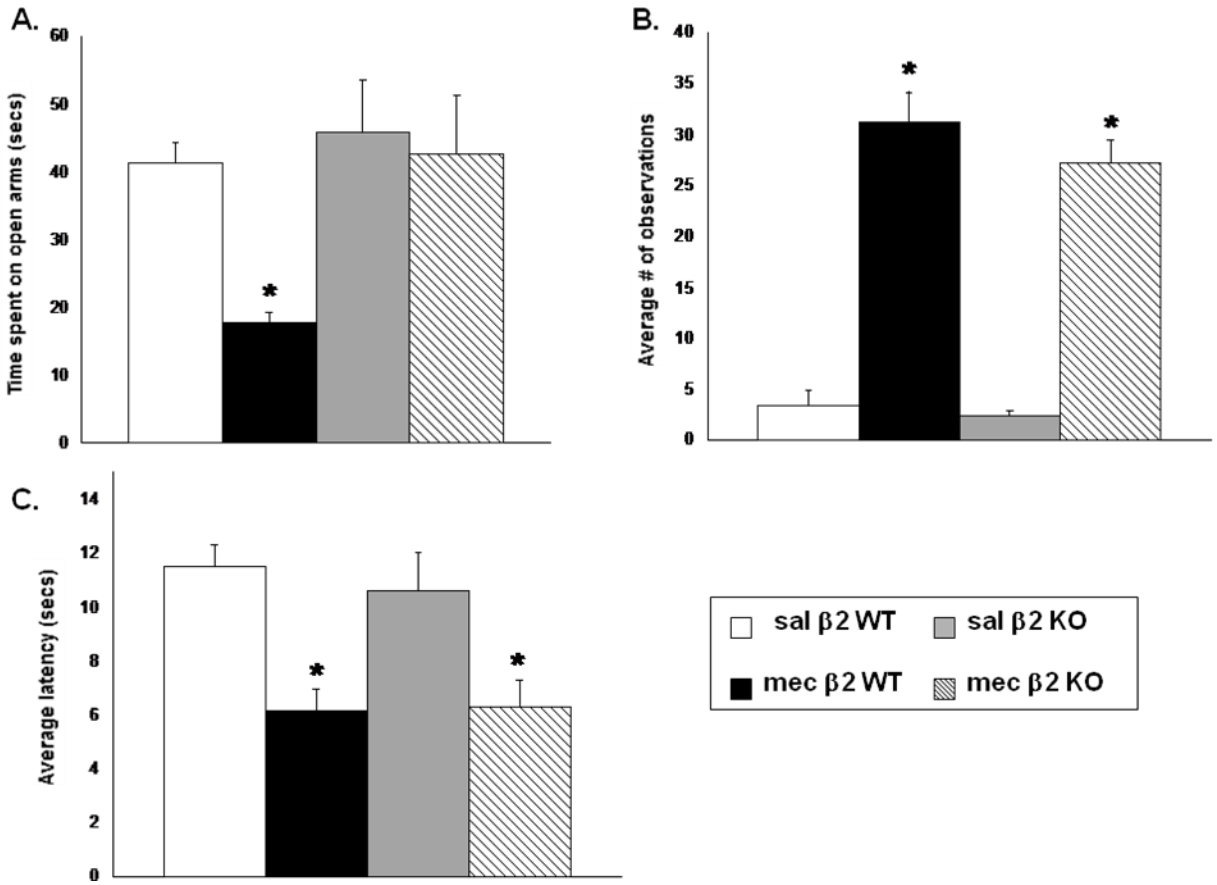


Figure 8. The β2 nAChR subunit is involved in the affective signs, but not the physical signs of nicotine withdrawal. When compared to WT chronic nicotine infused mice, chronic nicotine infused β2 KO mice show A. no reduction in the time spent on the open arms of the plus maze, indicating a loss of anxiety-related behavior, but B. significant nicotine withdrawal somatic signs and C. a decreased hotplate latency, indicating the presence of the hyperalgesia response. Each point represents the mean ± S.E.M. of 8 mice per group. * denotes $p < 0.05$ vs. saline groups and vs. nicotine KO group for the plus maze test.

Table 2: <u>Total average number of arm crosses in the plus maze test after precipitated nicotine withdrawal in β2 and α5 KO mice.</u>

Using the precipitated model, chronic nicotine infused mice were treated with mecamylamine (2 mg/kg, s.c.) on test day (day 15), and the total number of crosses between open and closed arms of the plus-maze test was counted. Numbers are presented as the total average number of arm crosses ± SEM for 8 mice per group.

β2 mice	**Sal-WT**	**Sal- KO**	**Mec-WT**	**Mec-KO**
	3.6 ± 0.59	3.6 ± 0.32	4 ± 0.46	3.3 ± 0.59
α5 mice	**Sal –WT**	**Sal- KO**	**Mec-WT**	**Mec-KO**
	2.8 ± 0.51	3 ± 0.49	3.3 ± 0.42	3.2 ± 0.40

Assessment of β2 nAChR KO mice in a spontaneous withdrawal model

It was important to demonstrate that the precipitated nicotine withdrawal behaviors observed in transgenic mice were not an assessment of the mecamylamine-dependent behavioral effects on specific nAChR subunits. Therefore, we used the spontaneous withdrawal model to assess nicotine withdrawal signs in β2 KO mice 18-24 hours after withdrawal from nicotine. Results of the spontaneous withdrawal assessment in β2 KO mice are shown in Figure 9. As observed in the mecamylamine-precipitated model, nicotine- withdrawn β2 KO mice displayed significant somatic signs and hyperalgesia, but a loss of anxiety-related behavior after cessation of nicotine treatment.

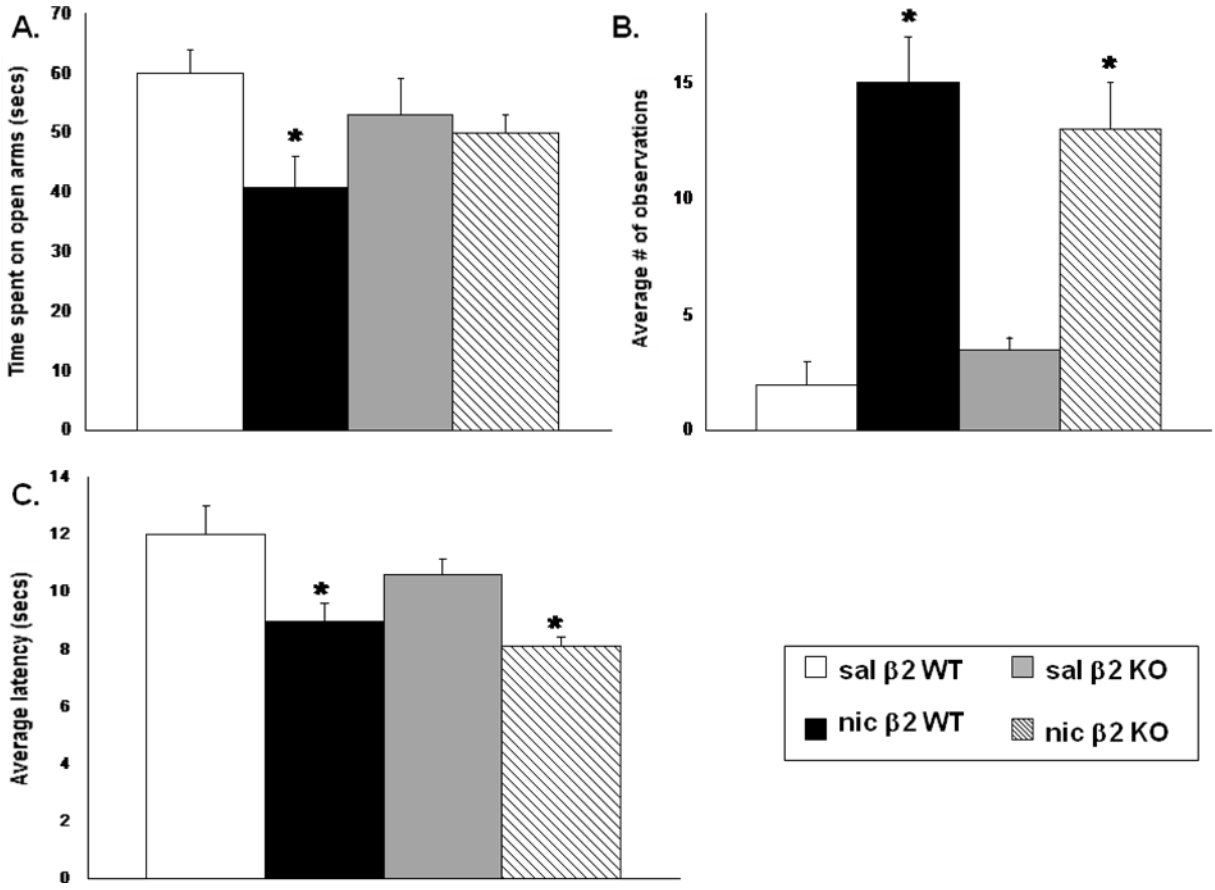


Figure 9. Assessment of nicotine withdrawal signs in β2 nAChR KO mice using the spontaneous withdrawal model. β2 KO mice withdrawn from nicotine 18-24 hours show A. a loss of anxiety-related behavior on the plus maze, indicated by the lack of a reduction in the time spent on the open arms, but B. significant somatic signs, and C. withdrawal-induced hyperalgesia, indicated by a decreased hotplate latency. Each point represents 8 mice per group. * denotes p< 0.05 vs. saline groups.

Role of the α5 nAChR subunit in nicotine withdrawal

The evaluation of nicotine withdrawal in α5 KO mice is shown in figure 10. Chronic nicotine infused WT and α5 KO mice spent significantly less time on the open arms of the elevated plus maze compared to saline infused WT and KO mice, indicating the presence of an anxiety-related response (Fig. 10A, Table 2). Somatic sign observation of chronic nicotine infused α5 KO mice revealed a significant reduction in paw tremors, backing, and total somatic signs compared to chronic nicotine infused WT littermates (Fig. 10B) Both WT and α5 KO chronic nicotine infused mice displayed a decreased latency on the hot plate, indicating a significant hyperalgesia response (Fig. 10C). There were no significant differences between saline infused KO and WT mice for any withdrawal test.

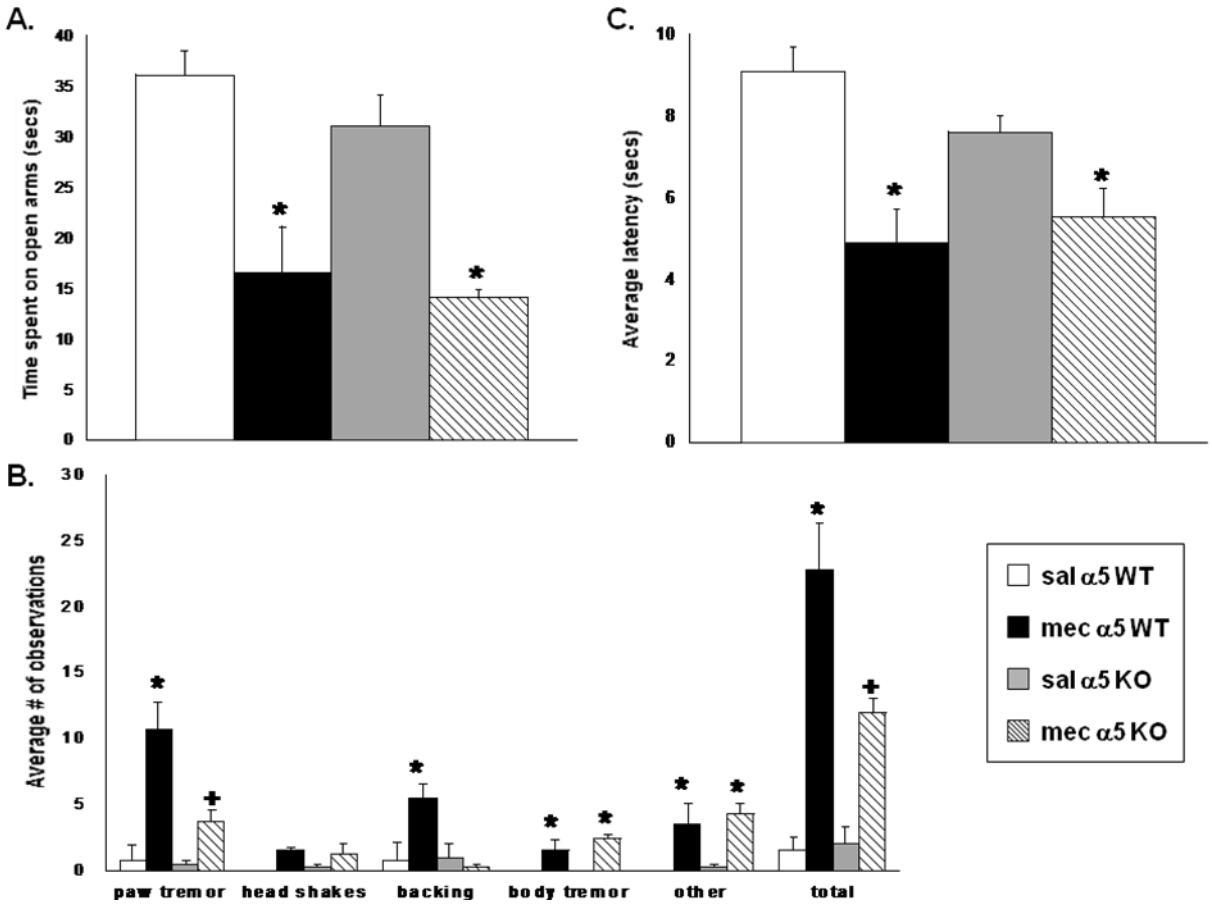


Figure 10. The α5 nAChR subunit plays a role in some physical aspects of nicotine withdrawal, but not the affective signs. Nicotine-dependent α5 KO mice show A. significant anxiety-related behavior in the plus maze test, indicated by a reduction in time spent on the open arms, but B. display a significant reduction in total average number of somatic signs when compared to WT counterparts. C. The nicotine-induced hyperalgesia response is still present in α5 KO mice, indicated by a decreased hotplate latency. Each point represents the mean ± S.E.M. of 6-8 mice per group. * denotes $p < 0.05$ vs. saline groups. + denotes $p < 0.05$ vs. saline groups and vs. WT mec group

Role of the α6 nAChR subunit in nicotine withdrawal

The role of α6*nAChRs in physical and affective nicotine withdrawal signs was evaluated using a spontaneous withdrawal model. As expected, nicotine withdrawn mice, compared to saline infused mice, spent significantly less time on the open arms of the plus maze, indicating withdrawal-associated anxiety-like behavior, exhibited decreased latency on the hot plate, indicating a withdrawal-induced hyperalgesia response, and displayed indications of somatic withdrawal (Fig. 11).

Pretreatment with the highest (30 pmol) dose of MII[H9A;L15A], blocked the expression of an anxiety-related response in nicotine-withdrawn mice. There was no significant difference in time spent on the open arms of the plus maze between saline infused mice and animals infused with MII[H9A;L15A] (Fig. 11A). The average number of arm crosses was tallied as a measure of locomotor activity on the plus maze. There was no significant difference between mice treated with vehicle and 30 pmol MII[H9A;L15A], suggesting that the observed effects on the plus maze were not the result of a difference in activity (veh= 2.4 ± 0.3 vs. 30 pmol= 2.4 ± 0.7, p=0.91).

Somatic withdrawal assessment revealed that nicotine-withdrawn mice treated with vehicle, 22.5 or 30 pmol MII[H9A;L15A] i.c.v. displayed significantly more total somatic signs than saline infused mice, indicating that MII[H9A;L15A] had no effect on somatic withdrawal at any dose tested (Fig. 11B). The hot plate assessment also revealed a significant hyperalgesia response in all nicotine-withdrawn mice compared to saline

exposed mice, regardless of i.c.v. injection, indicating that MII[H9A;L15A] had no effects on the nicotine withdrawal-induced hyperalgesia response at the doses tested (Fig. 11C). Saline infused mice treated with 30 pmol MII[H9A;L15A] i.c.v. did not differ from saline infused mice treated with vehicle i.c.v. in any behavioral test, indicating that the antagonist at the highest dose used was not behaviorally active by itself (Fig. 11).

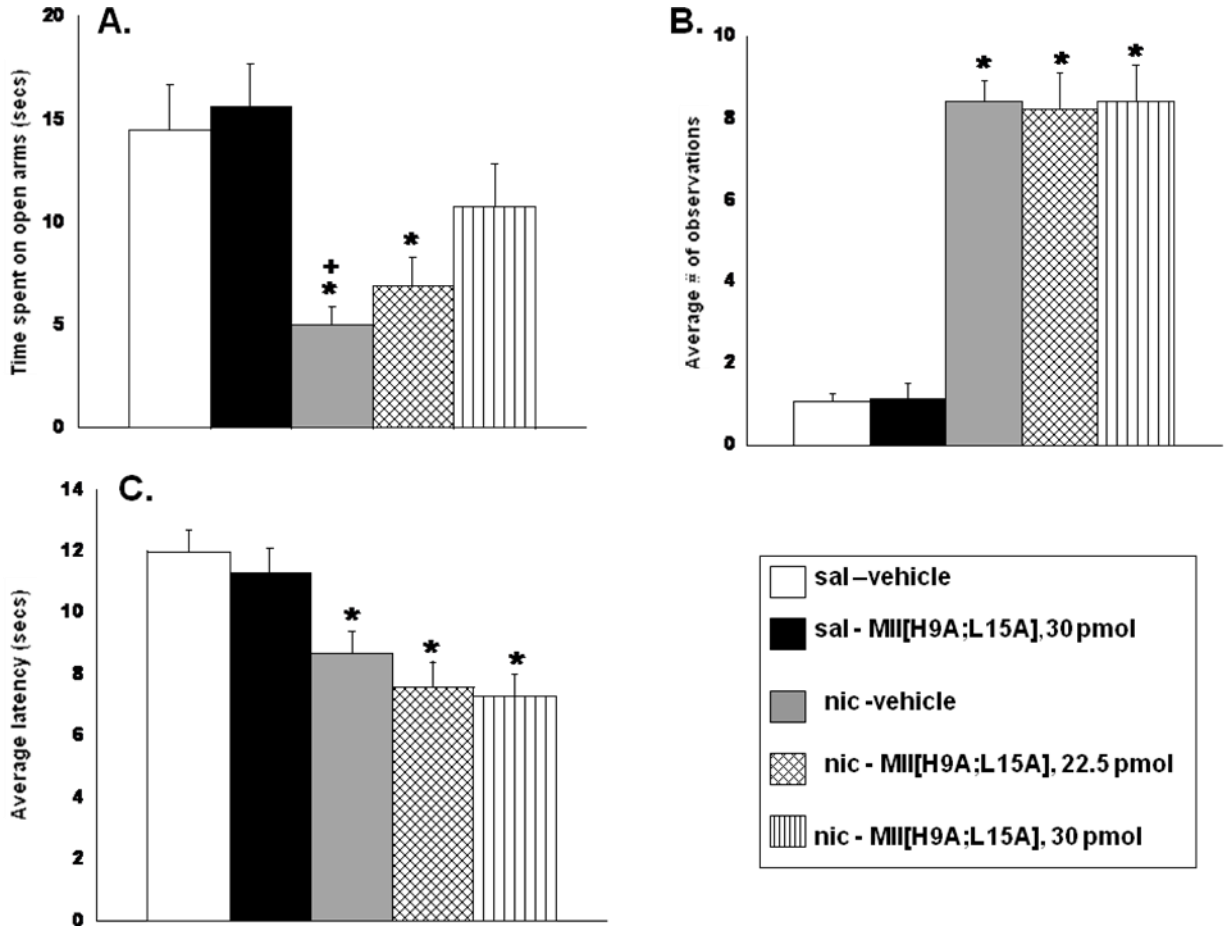


Figure 11. MII[H9A;L15A] dose-dependently blocks expression of the nicotine withdrawal-induced anxiety-related response in mice. Nicotine withdrawn mice treated with 30 pmol MII[H9A;L15A] display A. a loss of anxiety-related response, but B. no change in average number of somatic signs or C. hyperalgesia response. Each point represents the mean ± S.E.M. of 12 mice per group. * denotes p< 0.05 vs. saline groups; + denotes p< .05 vs. 30 pmol MII[H9A;L15A]

Role of the β2, α5, and α6 nAChR subunits in affective signs using the CPA model

In Chapter 2, we adapted a nicotine CPA model to further assess affective withdrawal signs in transgenic mice. Our CPA assessment using β2 and α5 nAChR KO mice, and the α6 selective antagonist MII[H9A;L15A], are shown in Figures 12 and 13 respectively. There was a loss of significant CPA in chronic nicotine infused β2 KO mice (Fig. 12A). In contrast, mecamylamine precipitated CPA was present in chronic nicotine infused α5 KO mice (Fig. 12B). In the α6 assessment, mecamylamine precipitated CPA in chronic nicotine infused mice treated with vehicle (Fig. 13). MII[H9A;L15A], however, dose-dependently blocked expression of aversion in nicotine-infused mice. Pre-treatment with 30 pmol, but not 22.5 pmol MII[H9A;L15A], on test day blocked the expression of mecamylamine-precipitated CPA in nicotine-dependent mice (Fig. 13).

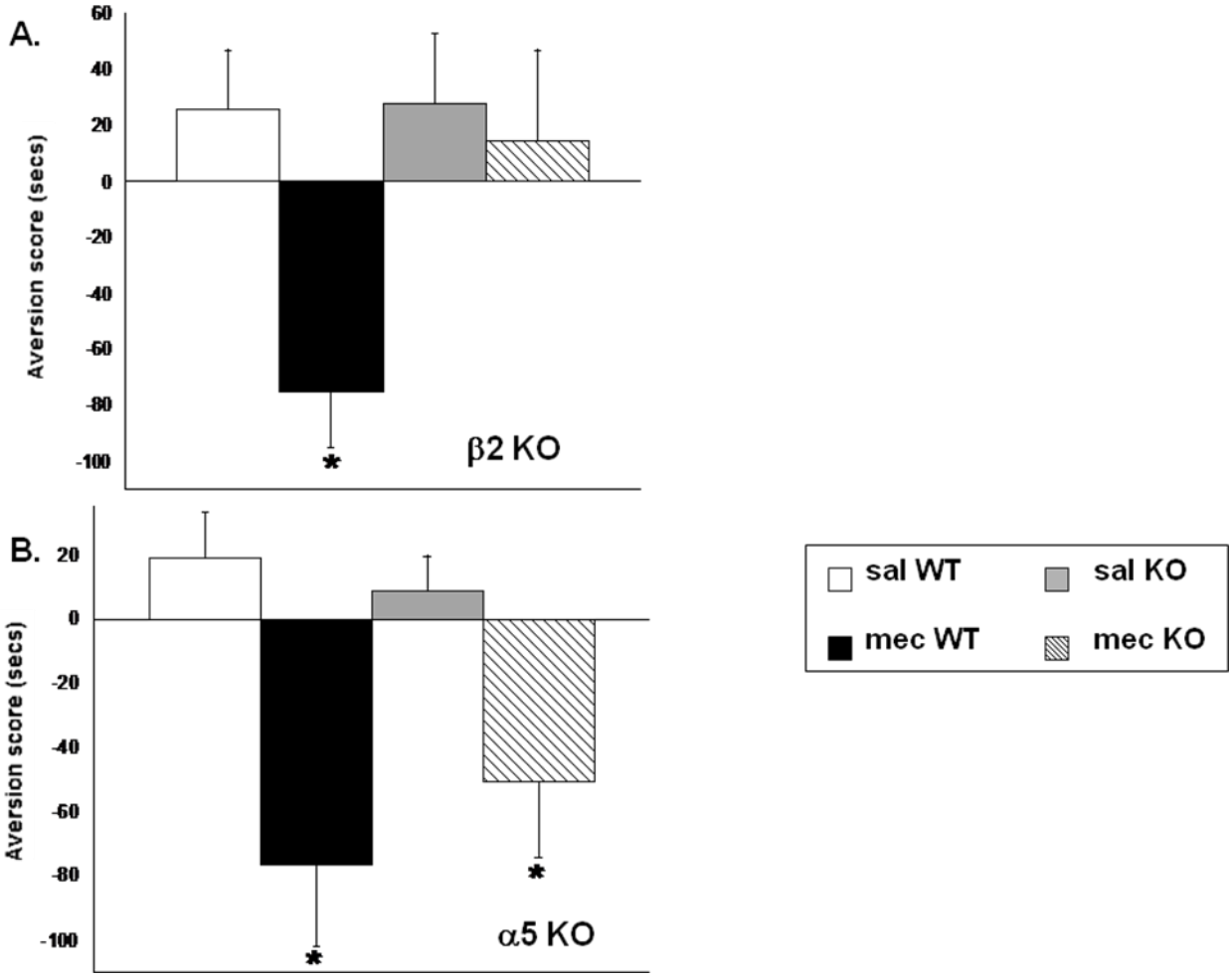


Figure 12. Assessment of the role of the β2 and α5 nAChR subunits in affective nicotine withdrawal signs using the CPA model. A. Nicotine-dependent β2 KO mice show a loss of significant CPA; however, B. α5 nAChR KO mice express mecamylamine-precipitated CPA. Each point represents the mean ± S.E.M. of 8-10 mice per group. * denotes $p < 0.05$ vs. saline groups and vs. mec KO group in the β2 assessment.

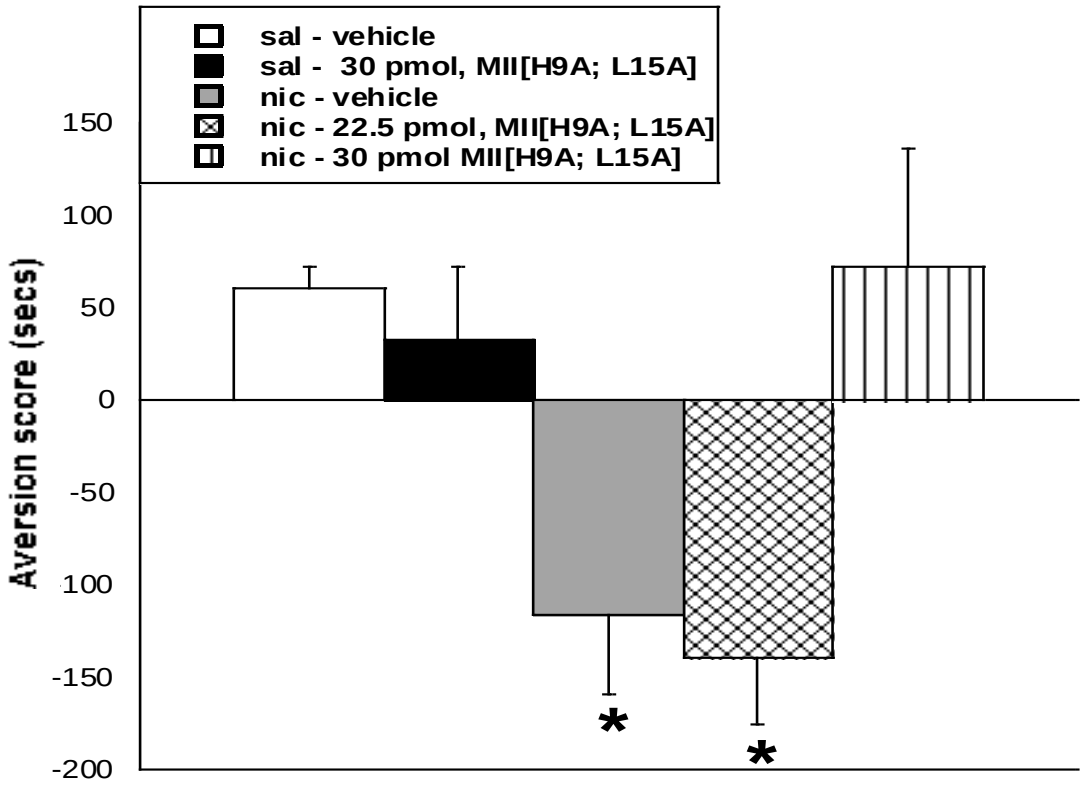


Figure 13. Assessment of the role of the α6 nAChR subunit in affective nicotine withdrawal signs using the CPA model. Nicotine-infused mice treated with 30, but not 22.5 pmol MII[H9A;L15A], did not express significant CPA. The 30 pmol dose of MII[H9A;L15A] had no effect in saline treated mice. Each point represents the mean ± S.E.M. of 12 mice per group.* denotes p< 0.05 vs. saline and 30 pmol MII[H9A;L15A] treated groups.

D. Discussion

The major goal of this study was to determine the role of major nAChR subtypes in the physical and affective signs of nicotine withdrawal. Therefore, using precipitated, spontaneous, and CPA nicotine withdrawal models to measure both physical and affective signs of nicotine withdrawal, we found a loss of anxiety-related behavior and nicotine withdrawal aversion, but normal physical withdrawal in β2 KO mice and in mice after pre-treatment with the selective α6 antagonist, MII[H9A;L15A], suggesting that β2 and α6 containing nAChRs are involved in affective, but not physical nicotine withdrawal. Studies with α5 KO mice revealed a reduction in somatic signs, but normal hyperalgesia and affective withdrawal, suggesting a role for the α5 subunit in the somatic signs associated with nicotine withdrawal.

Before proceeding with our studies using KO mice, we adapted a nicotine withdrawal model that would allow us to measure both physical and affective nicotine withdrawal signs in one setting. Results showed that mecamylamine precipitates anxiety-related behavior, somatic signs, and hyperalgesia in chronic nicotine infused mice. The half-life of mecamylamine in the rodent is approximately 1 hour (Debruyne et al., 2003). We were able to effectively measure one affective and two physical nicotine withdrawal signs within this one hour time frame. Our spontaneous withdrawal test using transgenic mice also confirmed that the observed precipitated- withdrawal behavioral effects were not an assessment of mecamylamine-dependent effects on nAChR subunits.

Results suggest that β2 nAChRs are involved in affective, but not physical nicotine withdrawal. There was no reduction in somatic signs and hyperalgesia was present in nicotine-withdrawn β2 KO mice. These data are consistent with previous studies assessing the role of β2 nAChRs in somatic signs of nicotine withdrawal using β2 KO mice (Salas et al., 2004; Besson et al., 2006). Both studies found that mecamylamine precipitated nicotine withdrawal somatic signs in chronic nicotine infused β2 KO mice in a similar fashion to what was observed in nicotine-infused WT littermates; however, neither study measured affective signs in β2 KO mice. In the current study, we assessed two affective signs of nicotine withdrawal in β2 KO mice and found that β2 KO mice displayed a lack of anxiety-related behavior in the plus maze, as well as a lack of mecamylamine-precipitated aversion in the CPA model. Our studies using transgenic mice are complemented by previous pharmacological nicotine withdrawal studies which utilized the β2-selective antagonist, DHβE, to precipitate anxiety-related behavior and elevations in reward threshold, also measures of affective signs of nicotine withdrawal (Damaj et al., 2003; Bruijnzeel and Markou, 2004). Further, in the previous chapter, our pharmacological studies showed that DHβE dose-dependently precipitated aversion in the CPA model. Due to the issue of compensation in transgenic animals, the complementary pharmacological approach strengthens our interpretation of the current results. Taken together, these studies suggest an important role for β2-containing nicotinic receptors in affective nicotine withdrawal behaviors.

Central administration of the selective α6 antagonist, MII[H9A;L15A], had no effect on physical withdrawal, but dose-dependently blocked anxiety-related behavior in the plus maze, as well as expression of aversion in the CPA model. These results indicate a role for α6 receptors in affective, but not physical nicotine withdrawal behaviors. Interestingly, 22.5 pmol MII[H9A;L15A] had no effect on the expression of CPA; however, 30 pmol MII[H9A;L15A], a dose not more than half, significantly blocked the effect. Currently the *in vivo* pharmacokinetics of MII[H9A;L15A] are not known; thus, it is possible that, like nicotine, the drug has a sharp dose response curve and produces its effects at a narrow range of concentrations. The LC, in addition to the VTA, has abundant α6 receptor expression, and both areas have been implicated in withdrawal from drugs of abuse. Studies show that intra-LC injections of a D1 receptor agonist attenuated morphine withdrawal signs in rats (Dizgah et al., 2005). Further, the α6* nAChR is expressed with the α4β2* subtype (Wada et al., 1989, 1990; Klink et al., 2001), and the current study, along with previous studies, suggests that β2*nAChRs are critical for affective, but not physical withdrawal (Damaj et al., 2003; Besson et al., 2006; Jackson et al., 2008). Taken together, these results suggest a role for the α4α6β2* nAChR subtype in affective nicotine withdrawal.

The role of the α5 nAChR subunit in nicotine withdrawal has not previously been addressed. Assessment of the α5 subunit showed a reduction in somatic signs in chronic nicotine infused α5 KO mice after mecamylamine treatment, while anxiety-related behavior and hyperalgesia were still present. Additional evaluation of affective signs

revealed the expression of mecamylamine-precipitated aversion in α5 KO mice. These findings suggest that the α5 subunit is involved to an extent in some physical aspects of nicotine withdrawal, but not affective nicotine withdrawal signs. It has been reported that the β4 subunit is involved in the physical signs of nicotine withdrawal, as nicotine-infused β4 KO mice displayed a significant reduction in somatic signs and a significant loss of hyperalgesia in the tail-flick, but not the hot plate assessment (Salas et al., 2004). Spinal and supraspinal nAChR populations mediate the hyperalgesia response (Schmidt et al., 2001; Damaj et al., 2002). While the hot plate assessment involves supraspinal receptor populations, the tail-flick test measures responses from spinal receptor populations. The β4 subunit is coexpressed with the α3 and α5 subunits in the peripheral ganglia (Salas et al., 2004). This may explain why there was an effect for β4 in the tail-flick, but not the hot plate assessment, as well as why the hyperalgesia response was present using the hot plate in our α5 assessment. Somatic signs of nicotine withdrawal were also shown to be partially mediated by peripheral nAChR populations; therefore, we propose that ganglionic α5β4*-containing nAChR subtypes mediate somatic signs of nicotine withdrawal. This hypothesis is evaluated further in the next chapter. Because the α5 subunit can co-assemble with both β2 and β4 nAChRs, it is likely that the role of α5 in nicotine withdrawal differs depending on nAChR subunit composition.

As mentioned, one of the problems in interpretation of results obtained with KO mice is whether compensatory changes in expression of other genes occur as a result of deletion of a particular gene. Although this issue has not yet been directly explored with

the nicotinic KO mice, it should be noted that behavioral differences observed with these KO mice were reproduced using various nicotinic antagonists, at least for the α7 and β2 subunits, as seen in the next chapter and previous chapter respectively, as well as with previous studies from the lab (Damaj et al., 2003). In addition, no compensatory changes of other nicotinic subunits were reported in these particular KO strains (Orr-Urtreger et al., 1997; Salas et al, 2004; Picciotto et al., 1995). Although we cannot completely rule out effects on other systems, these findings argue against a role for compensatory changes and suggest a direct role for these subunits in nicotine withdrawal.

Additionally, the issue of selectivity of nAChR agonists and antagonists has also produced some difficulty in data interpretation. While several α-conotoxins have affinity for both α3 and α6 nAChR subunits, MII[H9A;L15A] is a much more potent at α6* than at the α3* nAChRs (McIntosh et al., 2004). In the current study, we injected 22.5-30 pmol of peptide. Based on diffusion studies by Matta et al. (1995), the highest dose (30 pmol) would correspond to a tissue concentration of ~ 1.2 μM. At 1 μM, MII[H9A;L15A] has no effect on α2β2, α2β4, α4β2, α4β4, or α7 nAChRs. The peptide is also > 2000-fold selective for α6/α3β2β3 nAChRs compared to α3β2 and α3β4 nAChRs. The IC_{50} at α3β2 and α3β4 nAChRs is 4.8 μM and 7.8 μM respectively; thus, at concentrations used, there would be significantly less blockade of these receptor subtypes. The IC_{50} at α6/α3β2β3 and α6β4 nAChRs is 2.4 nM and 270 nM respectively (McIntosh et al., 2004); therefore, at the concentrations used in our studies, selective block of α6* nAChRs is expected.

The results of this study present a role for β2 and α6-containing nAChRs in affective nicotine withdrawal. In addition, the present findings indicate that the α5 subunit is not essential for affective withdrawal. Together with previous studies, these data suggest that α6β2* and/ or α4α6β2*, but not α4α5β2* nAChR subtypes, are critical for affective withdrawal. An examination of Ca^{2+}-dependent post-receptor mechanisms that contribute to these behavioral effects is addressed in Chapter 6.

IDENTIFICATION OF ADDITIONAL NICOTINIC RECEPTOR SUBUNITS INVOLVED IN NICOTINE WITHDRAWAL

A. Introduction

In the previous chapter, we began our identification of nAChR subunits involved in nicotine withdrawal with the β2, α5, and α6 nAChRs. We began our assessment with the β2 nAChR because it is a central, highly expressed subunit that has been implicated in several nicotine dependence behaviors, including reward and reinforcement (Picciotto et al., 1995; Walters et al., 2006). The α4, α5, and α6 nAChR subunits coassemble with β2 nAChRs to form functional receptors that are involved release of DA (Champtiaux et al., 2003; Salminen et al., 2004; Lai et al., 2005), a neurotransmitter involved in behaviors associated with nicotine dependence.

The homomeric α7 nAChR is also a major subtype found on neurons in the mesocorticolimbic drug pathway, and has been implicated in physical withdrawal behaviors. Pharmacologically, MLA, an α7 antagonist, was shown to precipitate mild somatic signs (Damaj et al., 2003) and changes in locomotor activity (Nomikos et al., 1999). Nicotine-withdrawn α7 KO mice also did not display withdrawal-induced hyperalgesia or decreases in locomotor activity (Grabus et al., 2005). A role for β4* nAChRs has also been suggested. It was shown that β4 KO mice do not display somatic signs or hyperalgesia after nicotine withdrawal (Salas et al., 2004). The α3 subunit is coexpressed with α5 and β4 nAChRs in the peripheral ganglia, MHb, and IPN (Wada et al., 1990; Zoli et al., 1995; Quick et al., 1999; Whiteaker et al., 2002). Further, α3 nAChR

subunits are expressed in brain areas that have been implicated as having a role in nicotine dependence behaviors, and can coassemble with β2 nAChRs to form functional receptors on DA neurons and terminals (Le Novère and Changeux, 1995; Le Novère et al., 1996; Klink et al., 2001). In Chapter 3, our assessment of α5 KO mice in withdrawal revealed that α5 KO mice display a significant reduction in somatic signs, but normal affective withdrawal, suggesting a role for this subunit in somatic nicotine withdrawal. Recent genetic studies suggest that a common haplotype in the CHRNA5/CHRNA3/CHRNB4 gene cluster, which codes for the α5, α3, and β4 subunits respectively, predisposes to nicotine dependence (Berrettini et al., 2008).

Evidence of a behavioral role for α7 and α3 nAChRs in nicotine dependence is still lacking; thus, the goal of this research was to determine the role of α7 and α3 nAChRs in nicotine withdrawal behaviors. Using α7 KO mice, the α6/α3β2* selective antagonist α-conotoxin MII (MII) , and the α3β4* selective antagonist α-conotoxin AuIB (AuIB), we questioned α7, α3β2*, and α3β4* nAChR contributions to nicotine withdrawal using the precipitated, spontaneous, and CPA models. Because the α3 subunit has been shown to coassemble with both β2 and β4 nAChRs in the brain, the use of two antagonists, which differentiate between α3β2* and α3β4* nAChR subtypes , would also allow us distinguish between α3 coassembly with the β4 and β2 nAChR subunits in nicotine withdrawal.

B. Methods

Animals

Male B6 mice were purchased from Jackson Laboratories. Mice lacking the α7 subunit of the nicotinic receptor (B6 background) and WT littermates were purchased from Jackson Laboratories (B6.129S7-charna7tm1bay, number 003232; see Orr-Urtreger et al., 1997 for information regarding initial breeders). For all experiments α7 KO mice were backcrossed to at least 8-10 generations and maintained on a breeding scheme as discussed in Chapter 3.

Drugs

(-)-Nicotine hydrogen tartrate salt and mecamylamine hydrochloride were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). The α6/α3β2*-selective antagonist MII and the α3β4*-selective antagonist AuIB were synthesized as described in Cartier et al., 1996 and Luo et al., 1998 respectively. The compounds were dissolved in physiological saline (0.9% sodium chloride) and administered to each animal by i.c.v. injection. The highest doses for MII (8 pmol) and AuIB (18 pmol) i.c.v. injections were calculated based on the functional IC_{50}s of each at its respective subtypes (Cartier et al., 1996; Luo et al., 1998).

Chronic nicotine administration

Mice were implanted with Alzet osmotic mini pumps [model 2002 (14 days) or model 2004 (28 days) Durect Corporation, Cupertino, CA] filled with saline or (-)-nicotine (36 mg/kd/day) as described in Chapter 2.

I.c.v. surgery

I.c.v. surgery and injection was performed as described in Chapter 3. Briefly, a scalp incision was made to expose the bregma, and unilateral injection sites were prepared using a 26-gauge needle with a sleeve of PE tubing to control depth of the needle at a site 2 mm rostral and 2 mm lateral to the bregma at a depth of 2 mm. On the morning of testing, a volume of 5 µl using a 26-gauge needle with a sleeve of PE tubing was injected into the lateral ventricle and held in place for 20 seconds to ensure drug delivery.

Nicotine withdrawal assessment

Studies using α7 KO mice were conducted using the precipitated withdrawal assessment as described in Chapter 3. In brief, α7 KO mice were treated with mecamylamine (2 mg/kg, s.c.) on the morning of day 15, 10 minutes before testing. Studies using MII and AuIB were conducted using the spontaneous withdrawal model as described in Chapter 3. Mice were injected i.c.v. with vehicle, MII (2.5 or 4 pmol), or AuIB (15 or 18 pmol) on the morning of day 15, 18-24 hours after mini pump removal, and withdrawal signs were measured 5 minutes after injection. Spontaneous withdrawal experiments were conducted using the same testing scheme as mentioned with precipitated studies.

Nicotine CPA

α7 assessment

Mecamylamine-precipitated CPA for α7 KO mice was conducted using male KO and WT littermates as described in Chapter 2.

α3β2 and α3β4* assessment*

After the mecamylamine conditioning session on day 3, i.c.v. injection sites were prepared. On day 4, mice received i.c.v. injections of vehicle, MII (2.5 or 4 pmol), or AuIB (15 or 18 pmol), 5 minutes before being placed in the test chambers.

Statistical analysis

For all data, statistical analyses were performed using StatView ® (SAS, Cary, NC, USA). Studies using transgenic mice were analyzed with two-way ANOVAs [with genotype and treatment as between subject factors] using the Neuman-Keuls post-hoc test. *p* values of less than 0.05 were considered significant.

C. Results

Role of the α7 nAChR subunit in nicotine withdrawal

The role of α7 nAChR receptors in nicotine withdrawal is shown in Figure 14. Chronic nicotine infused WT and α7 KO mice spent significantly less time on the open arms of the plus maze than saline infused mice, indicating the presence of an anxiety-related response (Fig. 14A). No significant difference in the number of arm crosses between groups was noted (Table 3). Significant withdrawal somatic signs were also observed in chronic nicotine infused WT and α7 KO mice (Fig. 14B). Significant hyperalgesia was observed in chronic nicotine infused WT mice after mecamylamine treatment; however, chronic nicotine infused α7 KO mice showed a loss of nicotine-withdrawal induced hyperalgesia, indicated by no significant decrease in hot plate latency compared to saline controls (Fig. 14C). Saline infused KO mice did not differ from WT counterparts in any withdrawal test.

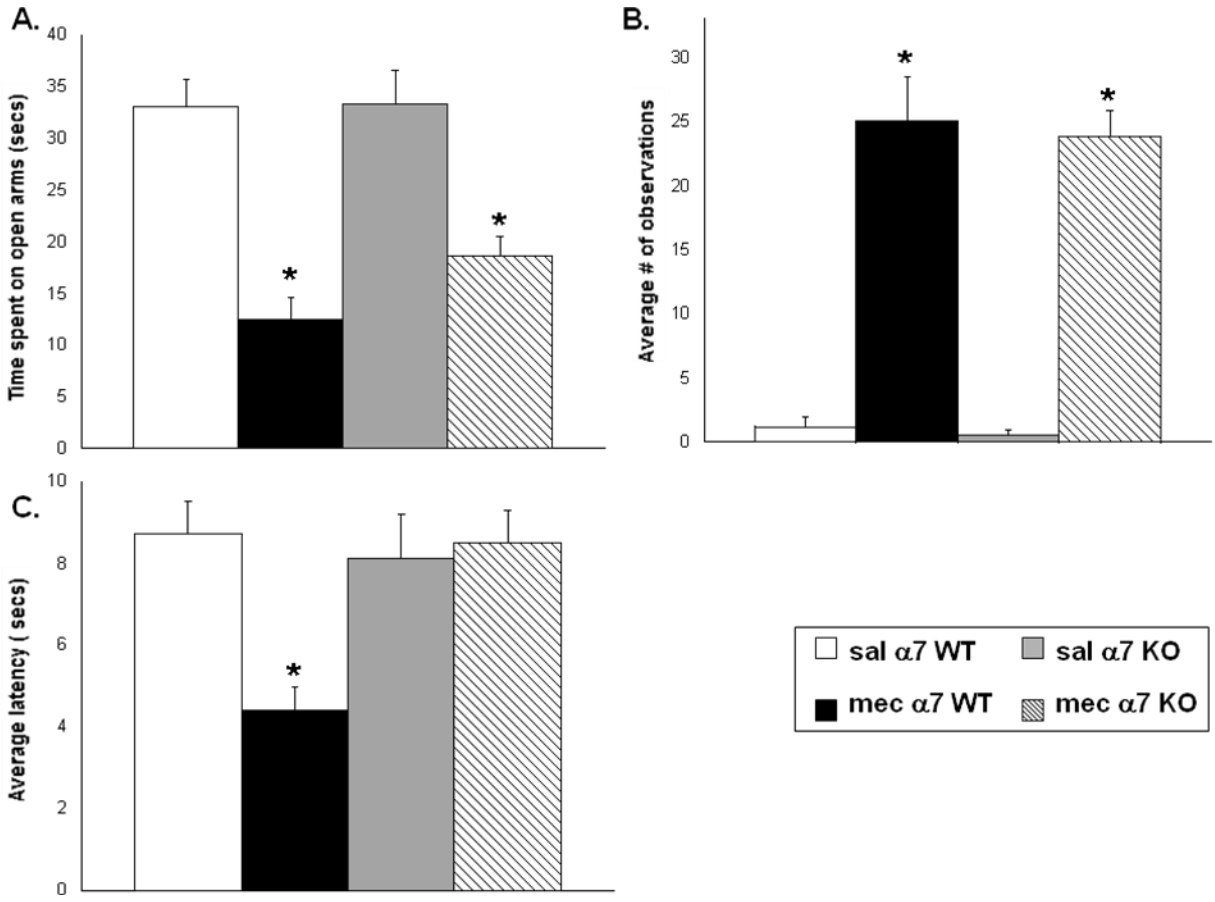


Figure 14. The α7 nAChR subtype is involved in some physical aspects of nicotine withdrawal, but not the affective signs. Chronic nicotine infused α7 KO mice showed A. a reduction in the time spent on the open arms of the plus maze, indicating an anxiety-related response, and B. significant nicotine withdrawal somatic signs. However, C. a loss of nicotine withdrawal-induced hyperalgesia response, as noted by the lack of a decrease in hotplate latency, was observed in chronic nicotine infused α7 KO mice when compared to WT counterparts. Each point represents the mean ± S.E.M. of 8 mice per group. * denotes p< 0.05 vs. saline groups and vs. nicotine KO group for the hyperalgesia test.

Table 3: Total average number of arm crosses in the plus maze test after precipitated nicotine withdrawal in α7 KO mice.

Using the precipitated model, chronic nicotine infused mice were treated with mecamylamine (2 mg/kg, s.c.) on test day (day 15), and the total number of crosses between open and closed arms of the plus-maze test was counted. Numbers are presented as the total average number of arm crosses ± SEM for 8 mice per group.

α7 mice	Sal-WT	Sal- KO	Mec-WT	Mec-KO
	2.7 ± 0.42	3.5 ± 0.48	3 ± 0.53	2.7 ± 0.28

Assessment of α7 nAChR KO mice in a spontaneous withdrawal model

It was important to demonstrate that the precipitated nicotine withdrawal behaviors observed in transgenic mice were not an assessment of the mecamylamine-dependent behavioral effects on specific nAChR subunits. Therefore, we used the spontaneous withdrawal model to assess nicotine withdrawal signs in α7 KO mice 18-24 hours after withdrawal from nicotine. Spontaneous withdrawal studies using α7 KO mice were comparable to mecamylamine-precipitated studies as shown in Figure 15. Nicotine-withdrawn α7 KO mice showed significant somatic signs and anxiety-related behavior, but no withdrawal-induced hyperalgesia response.

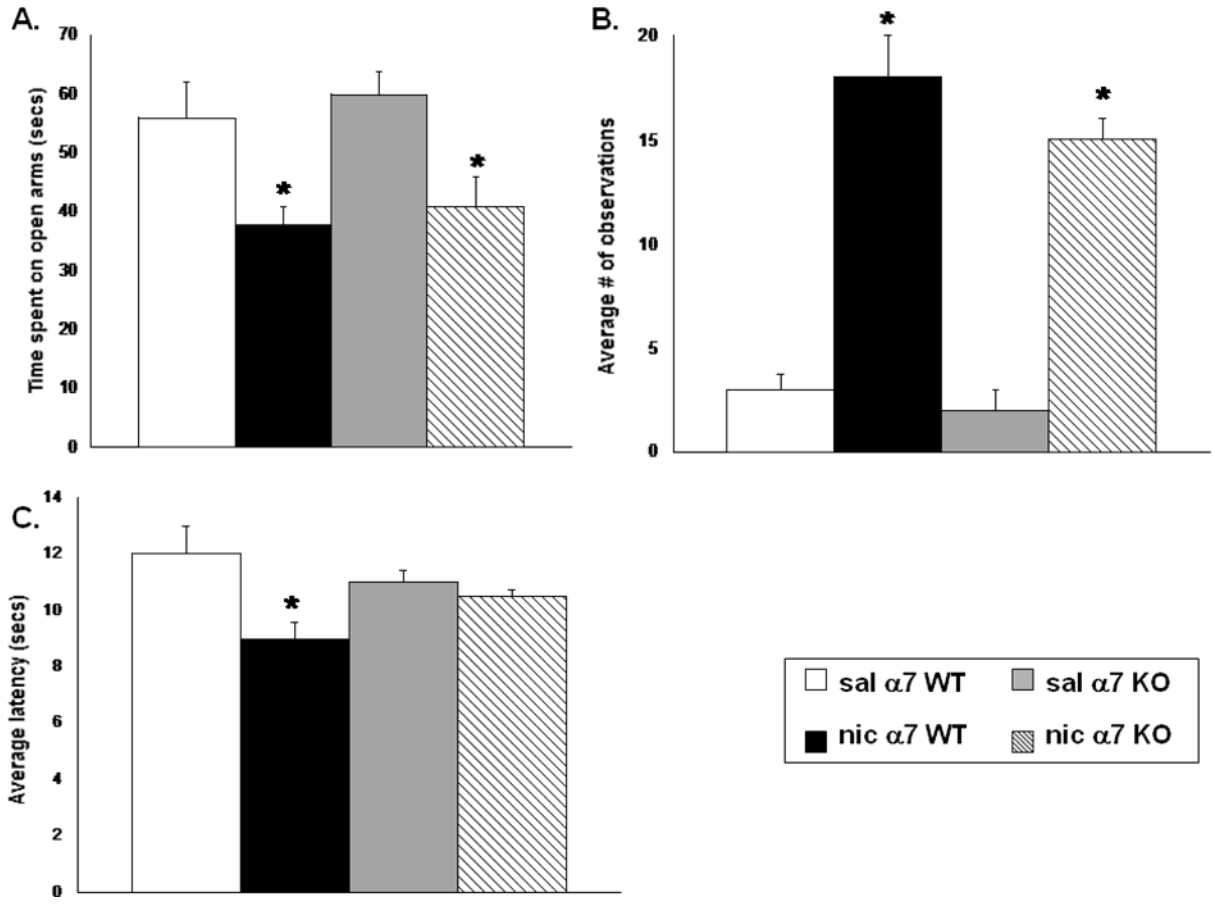


Figure 15. Assessment of nicotine withdrawal signs in α7 nAChR KO mice using the spontaneous withdrawal model. α7 KO mice withdrawn from nicotine 18-24 hours show A. anxiety-related behavior on the plus-maze, indicated by a reduction in the time spent on the open arms, and B. significant somatic signs, but C. a loss of withdrawal-induced hyperalgesia, noted by the lack of a decreased hotplate latency. Each point represents 8 mice per group. * denotes p< 0.05 vs. saline groups.

Role of α3β2* nAChRs in nicotine withdrawal

To evaluate the role of α3β2* nAChRs in nicotine withdrawal, we used the α6/α3β2* selective antagonist, MII. In Chapter 3, the α6-selective antagonist, MII[H9A;L15A], was used to evaluate the role of α6-containing nAChRs in nicotine withdrawal. MII[H9A;L15A] is able to effectively discriminate between α3 and α6 nAChRs; thus, we used MII, which has significantly less selectivity for α6-containing nAChRs than MII[H9A;L15A] (McIntosh et al., 2004), to separate the α6 component, and assess the role of α3β2* nAChRs in nicotine withdrawal. Results show that MII dose-dependently blocked an anxiety-related response in nicotine-withdrawn mice (Fig. 16A). Nicotine-withdrawn mice treated with 4, but not 2.5 pmol MII, did not display a reduction in time spent on the open arms of the plus maze as observed in nicotine-withdrawn vehicle treated mice. The number of arm crosses was tallied and there was no significant difference in arm crosses between vehicle and MII (4 pmol, i.c.v.) treated saline infused mice (veh= 3.1 ± 0.7; MII= 2.3 ± 0.5, p= 0.26) and no significant difference in time spent on the open arms, suggesting that the highest does of MII was not behaviorally active by itself (Fig. 16A).

Somatic sign assessment revealed a dose-dependent attenuation of total somatic signs after MII treatment (Fig. 16B), while MII had no effect in the hyperalgesia response at any dose tested (Fig. 16C).

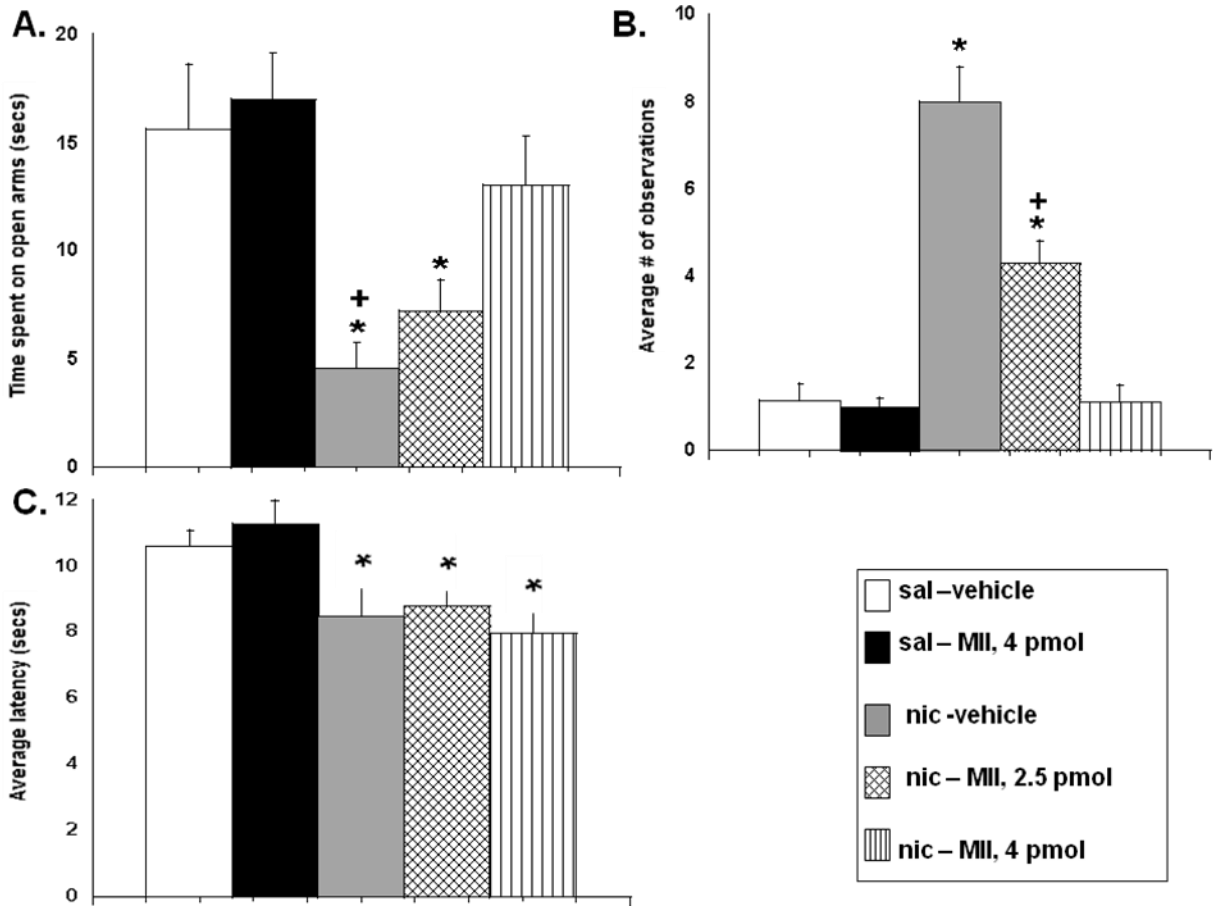


Figure 16. MII dose-dependently blocks expression of the nicotine withdrawal-induced anxiety-related response and somatic signs in mice. Nicotine withdrawn mice treated with 4 pmol, but not 2.5 pmol MII, display A. a loss of anxiety-related response, B. attenuation of somatic signs, but C. a normal hyperalgesia response. Each point represents the mean ± S.E.M. of 12 mice per group. * denotes p< 0.05 vs. saline groups; + denotes p< .05 vs. 4 pmol MII

Role of α3β4* nAChRs in nicotine withdrawal

The α3β4*-selective antagonist AuIB was evaluated in our nicotine withdrawal models. AuIB, purified from the venom of the "court cone", *Conus aulicus,* blocks the α3β4 receptor subtype with > 100 fold higher potency than other receptor combinations, such as α3β2 and α4β4 (Luo et al., 1998). Nicotine-withdrawn mice treated with vehicle i.c.v. displayed a significant anxiety-related response, and treatment with AuIB had no effect on the anxiety-related response at any dose tested (Fig. 17A). There was no significant difference in time spent on the open arms of the plus maze between any nicotine-withdrawn mouse group, regardless of i.c.v. treatment. Evaluation of saline infused mice revealed no significant difference in the amount of time spent on the open arms and no significant difference in the number of arm crosses between vehicle or AuIB treated mice (veh= 3.2 ± 0.4 ; AuIB= 2.7 ± 0.5, p= 0.41), suggesting that the highest dose of AuIB (18 pmol) was not behaviorally active alone.

In the somatic sign assessment, AuIB induced a dose-dependent decrease in total somatic signs. Nicotine-withdrawn mice treated with 15 pmol AuIB displayed a significant reduction in somatic signs compared to vehicle treated mice, while a dose of 18 pmol AuIB attenuated somatic signs in nicotine withdrawn mice (Fig. 17B). The hyperalgesia response was not affected by treatment with AuIB. Nicotine-withdrawn mice treated with saline or AuIB displayed a significant hyperalgesia response compared to saline infused animals, and there was no significant difference between nicotine withdrawn mice that received vehicle or AuIB (Fig. 17C).

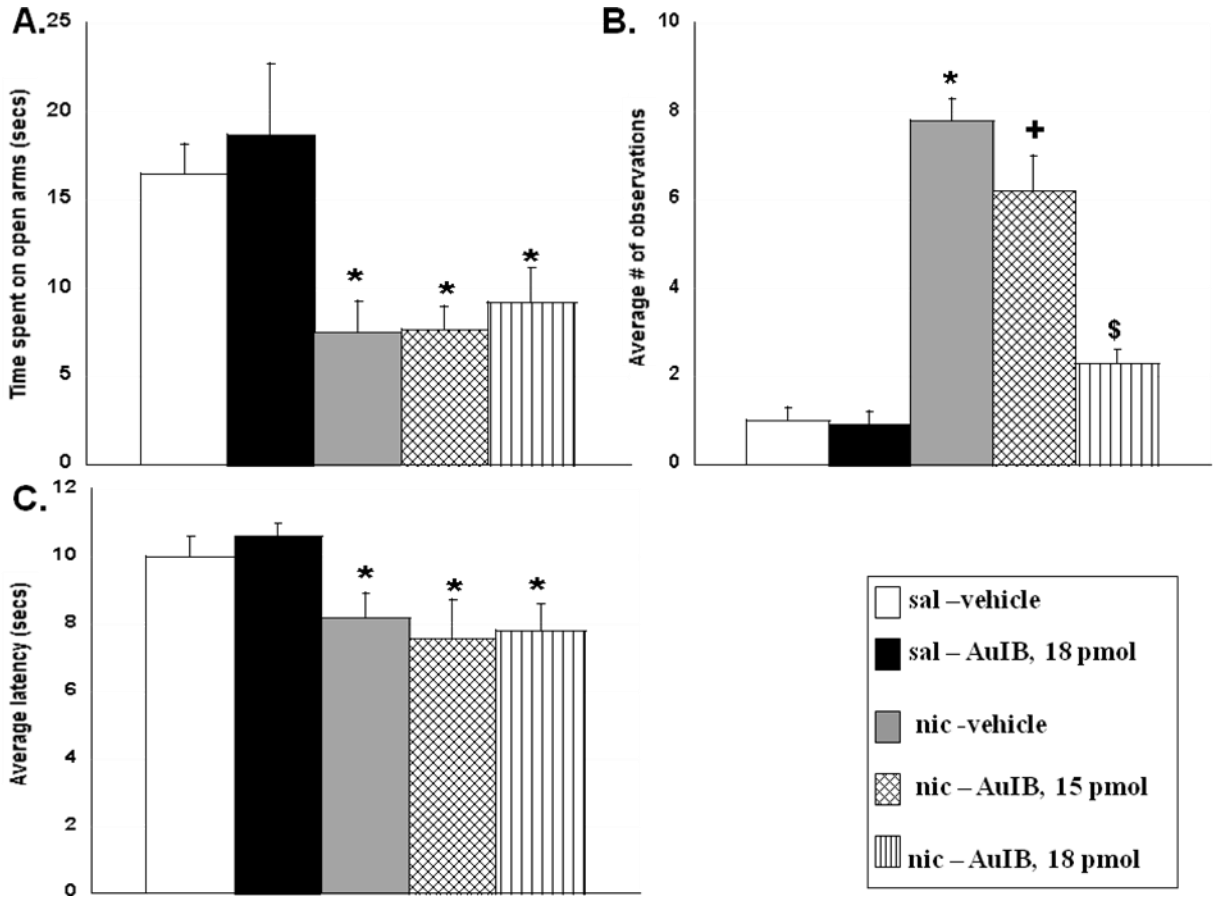


Figure 17. AuIB dose-dependently reduces somatic signs, but has no affect on anxiety-related behavior or the hyperalgesia response. Nicotine withdrawn mice treated with 18 pmol MII display A. no change in anxiety-related response or C. hyperalgesia response, but B. attenuation of total somatic signs. Each point represents the mean ± S.E.M. of 12 mice per group. * denotes p< 0.05 vs. saline groups; + denotes p< .05 vs. saline and nicotine-vehicle groups; $ denotes p< 0.05 vs. nicotine-vehicle and 15 pmol AuIB groups

Role of the α7, α3β2*, and α3β4*nAChR subtypes in affective signs using the CPA model

To further evaluate the role of the α7, α3β2*, and α3β4* nAChR subtypes in affective nicotine withdrawal, we tested α7 KO mice and mice after treatment with α3β2* and α3β4* in our CPA model. Our initial assessment suggested that α7* nAChRs were not involved in the affective withdrawal response. Results show that mecamylamine (3.5 mg/kg, s.c.) precipitated aversion in α7 WT and KO mice (Fig. 18). The CPA assessment also revealed that MII (4 pmol, i.c.v.) had no effect on expression of CPA on test day (Fig. 19A). Because our assessment in the plus maze revealed that MII blocked the anxiety-related response, we tested a higher dose of MII to determine if we could produce an effect. An 8 pmol dose of MII also had no effect on expression of CPA on test day (Fig. 19A). Nicotine infused mice treated with 18 pmol AuIB expressed a significant aversion similar to that observed in nicotine infused vehicle treated mice, indicating that expression of CPA was not blocked by AuIB (Fig. 19B). Saline infused mice treated with AuIB (18 pmol) or MII (8 pmol) did not significantly differ from saline infused vehicle treated mice, suggesting, by themselves, the highest doses of AuIB and MII used for the study were not behaviorally active.

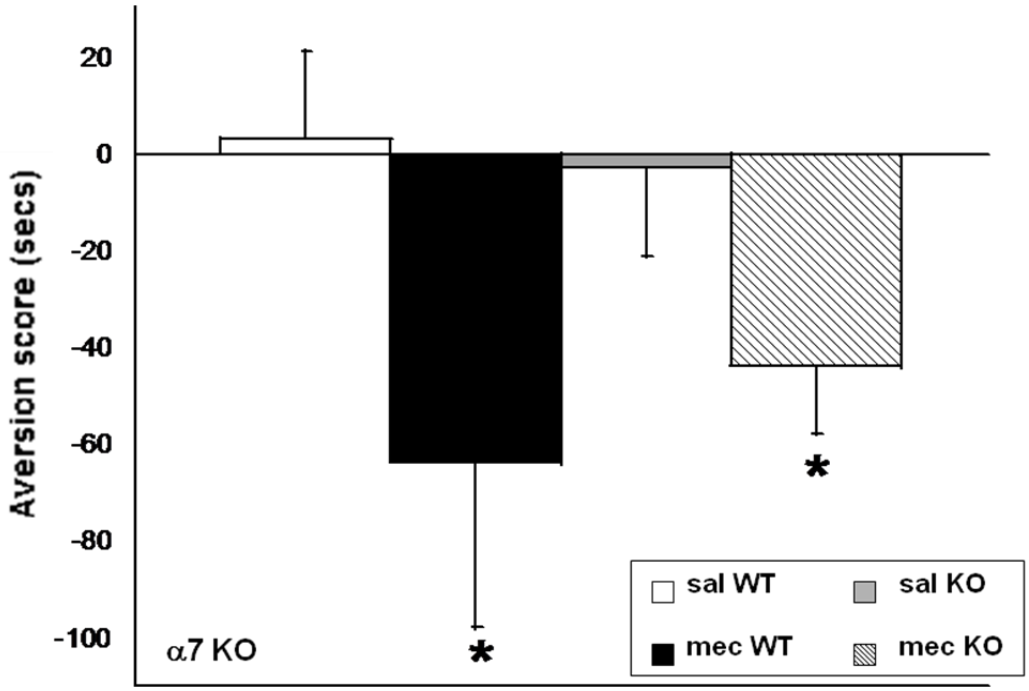


Figure 18. Mecamylamine precipitates aversion in nicotine-infused α7 WT and KO mice. Nicotine-infused KO mice treated with mecamylamine (3.5 mg/kg, s.c.) did not differ from their WT counterparts in the CPA assessment. * denotes p > 0.05 vs. saline counterparts.

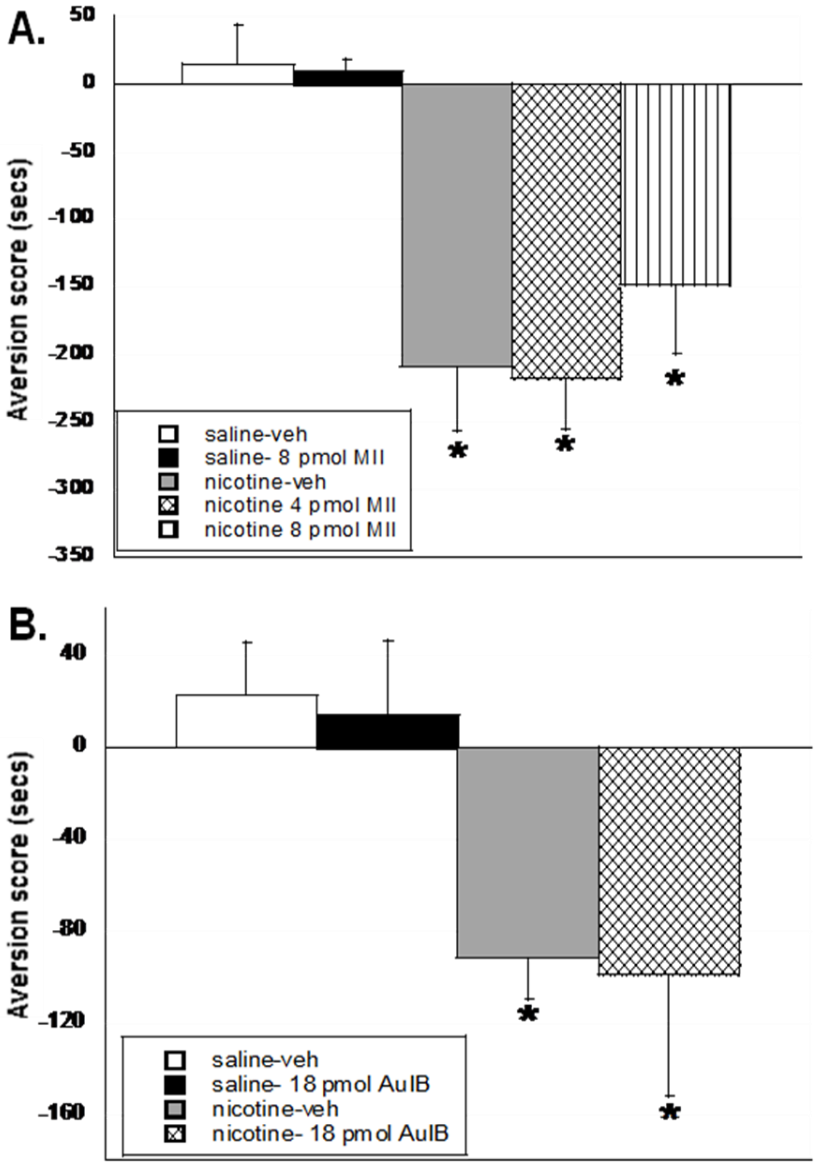


Figure 19. The α6/α3β2* selective antagonist MII and the α3β4* antagonist AuIB have no effect on expression of nicotine CPA. Mecamylamine precipitated CPA in chronic nicotine-infused mice; however, neither A. 8 pmol MII nor B. 18 pmol AuIB had an effect on expression of mecamylamine-precipitated aversion. Each point represents the mean ± S.E.M. of 12 mice per group.* denotes p< 0.05 vs. saline

D. Discussion

These behavioral studies using pharmacological agents and genetically modified mice suggest that α7* and α3β4* nAChRs are involved in physical nicotine withdrawal signs, while α3β2* nAChRs are involved in the somatic signs and anxiety-related response associated with nicotine withdrawal. Nicotine infused α7 KO mice displayed significant anxiety-related behavior, CPA, and somatic signs after mecamylamine injection, but a loss of the hyperalgesia response, suggesting a role for α7 in some physical, but not affective withdrawal. I.c.v. injection of the selective α6/α3β2* antagonist, MII, blocked the expression of anxiety-related behavior and somatic signs, but not expression of nicotine CPA, while treatment with the α3β4* antagonist only blocked the expression of somatic signs, suggesting that the α3β4* nAChR subtype is involved in somatic, but not affective withdrawal signs, while the α3β2* subtype appears to be involved in nicotine withdrawal-induced somatic signs and the anxiety-related response.

Assessment of the α7 nAChR subunit using transgenic mice showed that nicotine-dependent α7 KO mice expressed anxiety-related behavior, CPA, and somatic signs, but a loss of hyperalgesia. In the previous chapter, we found that MLA did not precipitate aversion in the CPA model in chronic nicotine infused mice, thus, complementing our results using transgenic animals. A previous α7 nAChR KO study from our laboratory using the oral route of chronic nicotine administration and the spontaneous nicotine withdrawal model produced similar findings (Grabus et al., 2005). The oral route of administration, however, yields variable amounts of nicotine intake, making it difficult to

control the actual dose of nicotine reaching the receptor; therefore, we wanted to assess the role of the α7 subunit using a more consistent exposure method. Taken together, these results suggest that α7 nAChRs are involved in physical, not affective, nicotine withdrawal signs.

The data also suggest that our two physical measures of nicotine withdrawal (somatic signs and hyperalgesia) are mediated by different nAChR subtype populations. While α7 KO mice showed a loss of the hyperalgesia response, these mice exhibited somatic signs of nicotine withdrawal. The α7 nAChR subunit is expressed in the peripheral ganglia, as well as centrally. Although hyperalgesia is measured as a physical sign in the mouse, studies have suggested that spinal and supraspinal nAChR populations mediate the hyperalgesia response (Schmidt et al., 2001; Damaj et al., 2002). Our hyperalgesia measure was conducted using the hot plate, which measures supraspinal mechanisms; thus, we cannot rule out the possibility that the nicotine withdrawal induced hyperalgesia response is mediated by neuronal α7 nAChRs. It is also noted that MLA, the α7 antagonist, precipitated mild somatic signs of withdrawal in WT mice (Damaj et al., 2003), while our assessment revealed the presence of typical nicotine withdrawal somatic signs in α7 KO mice. It was also shown, however, that MLA can antagonize α6, α3, and β3 nAChR subunits at doses typically utilized to block α7 nAChRs (Mogg et al., 2002); thus, it is possible that the observed behavioral responses were attributed to effects on other nAChR subtypes.

An i.c.v. injection of MII, the α6/α3β2* selective antagonist, dose-dependently blocked the expression of anxiety-related behavior and somatic signs, but not expression of CPA. One possible explanation as to why we saw an effect with MII in the plus maze, but not the CPA assessment is that anxiety-related behavior and CPA are mediated by two different brain regions; the dorsal raphé nucleus (Cheeta et al., 2001) and the basolateral amygdala (Zanoveli et al., 2007) respectively; thus, it is possible that the two behaviors are mediated by different subtypes. Furthermore, the α6 and α3 nAChR subunits share considerable sequence homology (Le Novère and Changeux, 1995). Indeed, studies suggest that MII may not discriminate well between α3 and α6 nAChRs (Kuryatov et al., 2000); however, the MII analog, MII[H9A;L15A], effectively discriminates between α3 and α6 nAChR subunits (McIntosh et al., 2004). Our results from Chapter 3 showed a role for α6-containing nAChRs in nicotine CPA and the anxiety-related response, but not physical withdrawal. These results suggest that α6-containing nAChRs, and not α3β2* nAChRs ,are important for nicotine CPA, while both α6 and α3β2* nAChRs are involved in the withdrawal-induced anxiety-related response; however, because MII does not discriminate well between α3 and α6 nAChRs, we cannot rule out the possibility that the effects of MII in the plus maze are attributed to actions at α6β2* nAChRs. Results using MII[H9A;L15A] showed that CPA was blocked at a dose of 30 pmol MII[H9A;L15A], but there was no effect at the 8 pmol MII dose used in the current study. Because MII[H9A;L15A] has higher selectivity for α6-containing receptors than MII, higher doses of MII may be required to block α6-containing receptors, and thus, expression of CPA. MII also dose-dependently blocked the expression of nicotine withdrawal somatic signs.

Although results from Chapter 2 showed that β2-containing receptors are not involved in somatic nicotine withdrawal signs, based on the current data, we cannot rule out the possibility that the α3β2* subtype is involved in this aspect of nicotine withdrawal.

The α3β4* selective antagonist, AuIB, given centrally, also dose-dependently attenuated somatic signs, but had no effect in the plus maze or CPA assessments. Studies using transgenic mice revealed an attenuation of somatic signs in β4 KO mice after mecamylamine-precipitated withdrawal (Salas et al., 2004). Somatic withdrawal signs were shown to be mediated by central, as well as peripheral nAChR populations by systemic and central administration of chlorisondamine, a noncompetitive nAChR antagonist that does not readily cross the blood brain barrier (Watkins et al., 2000). Chlorisondamine, however, is not selective for specific nAChR subtypes. Additionally, the β4 KO mouse used in the Salas et al. (2004) study is a general KO; thus, central and peripheral nAChR populations cannot be differentiated. Although the α3β4* subtype is both peripherally and centrally expressed, these studies suggest a role for central α3β4* nAChRs in somatic nicotine withdrawal. The α3β4* subtype has limited expression in the CNS, but was shown to dominate function in the MHb (Quick et al., 1999); therefore, α3β4* expression specifically in the MHb may be important for somatic nicotine withdrawal.

Based on studies from the previous chapter, and the current study, our data suggests that the α4α6β2* nAChR subtype is involved in affective nicotine withdrawal, while α7*, α5, and α3β4* nAChRs mediate physical nicotine withdrawal. Specifically, α7* nAChRs mediate the withdrawal induced hyperalgesia response, and the α5 and α3β4* subtypes

play a role in somatic withdrawal. These studies also stress the importance of receptor subunit composition in nicotine withdrawal. While β2-containing receptors were shown to be involved in affective, but not physical withdrawal, the α3β2* subtype was implicated in somatic nicotine withdrawal signs, and played no role in expression of nicotine CPA. The next chapter will focus on the involvement of intracellular Ca^{2+}-dependent mechanisms in the nicotine withdrawal syndrome.

THE ROLE OF CALCIUM-DEPENDENT MECHANISMS IN NICOTINE WITHDRAWAL

A. Introduction

Activation of nAChRs leads to increases in intracellular Ca^{2+} via various routes. Upon nicotine binding, there is a direct Ca^{2+} influx through the Ca^{2+} permeable nAChR. The resulting increase in intracellular Ca^{2+} leads to an indirect Ca^{2+} influx by Ca^{2+} induced Ca^{2+} release from intracellular Ca^{2+} stores, and through VGCC as a result of membrane depolarization following nAChR activation (Rathouz and Berg, 1994; Dajas-Bailador et al., 2002). The subsequent rise in intracellular Ca^{2+} leads to activation of various downstream second-messengers, including CaMKII, the most abundant Ca^{2+}-dependent kinase in the neuron (Deisseroth et al., 1998), and a protein involved in several essential processes, including neurotransmitter release (Schulman and Hanson, 1993) and induction of LTP (Lisman et al., 2002).

Studies suggest a role for L-type VGCCs and CaMKII in the effects of several drugs of abuse. L-type VGCCs are upregulated in the cortex and limbic structures of rats showing signs of morphine withdrawal (Antkiewicz-Michaluk et al., 1990; Zharkovsky et al., 1993) and blockade of L- type VGCCs attenuates signs of morphine physical dependence in rats (Michaluk et al., 1998; Vitcheva and Mitcheva, 2004; Esmaeili-Mahani et al., 2008). L-type VGCC blockers also inhibit chronic cocaine and amphetamine-induced behavioral and neurochemical changes (Karler et al., 1993; Pierce and Kalivas, 1997; Pierce et al., 1998). Additionally, blockade of L-type VGCC or suppression of CaMKII activity in the VTA augments acute cocaine-induced behavioral hyperactivity

(Licata et al., 2004). Nicotine studies show that pharmacological blockade of L-type VGCCs attenuates mecamylamine-precipitated somatic signs of nicotine withdrawal in mice (Biala and Weglinska, 2005). Studies from our lab showed that L-type VGCC blockers and CaMKII inhibitors block development and expression of nicotine-induced antinociception at the spinal level (Damaj, 2005). Biochemical studies show that L-type VGCCs, as well as α4β2* nAChRs, are upregulated in mouse cerebral cortical neurons after 7 days chronic nicotine exposure, leading to an increased Ca^{2+} influx (Katsura et al., 2002). Further, the signaling pathway that results in nicotine-induced ERK phosphorylation in mouse primary cortical neurons involves L-type VGCCs and CaMKII (Steiner et al., 2007). In PC12 cells, an increase in intracellular Ca^{2+} after stimulation of nicotinic receptors activates CaMKII (MacNicol and Schulman, 1992), and data from our lab shows that an acute systemic injection of nicotine is sufficient to elevate CaMKII in the spinal cord (Damaj, 2000). Recent studies revealed that alterations in the phosphorylation state of neuronal CaMKII occur after chronic exposure to various drugs of abuse such as cocaine, amphetamine and morphine (Tan, 2002; Wang Z et al., 2003; Licata et al., 2004). Although the current studies evaluating Ca^{2+}-dependent mechanisms in nicotine dependence provide evidence of a role for Ca^{2+}signaling, the behavioral studies only assess the physical aspect of nicotine withdrawal, and not the affective component, which is suggested to be of greater motivational significance in contributing to relapse (Koob et al., 1993; Markou et al., 1998). Additionally, there is no available behavioral correlation for the biochemical studies, which evaluate nicotine-induced Ca^{2+} mechanisms *in vitro*; thus, the relevance of these mechanisms to nicotine withdrawal is unclear. The goal of the

current study was, therefore, to elucidate the behavioral relevance of L-type VGCC and CaMKII in physical and affective nicotine withdrawal. Using our adapted spontaneous nicotine withdrawal and CPA models, mice were treated with one of two structurally different L-type VGCC blockers, nimodipine or verpamil, the L-type VGCC activator, (±) Bay K8644, the CaMKII inhibitor KN93, or its inactive analog KN92, and physical and affective withdrawal signs were measured. KN93 can also inhibit other kinases, such CaMKIV (Enslen et al., 1994); therefore, to complement our studies of CaMKII inhibition, we measured physical and affective nicotine withdrawal signs in nicotine-dependent CaMKII HT mice in our mecamylamine-precipitated model.

B. Methods

Animals

Male B6, male B6.129P2-Camk2atm1Sva/J HT, and female B6129P3 mice were purchased from Jackson Laboratories. CaMKII mutant mice were generated as described by Silva et al., 1992a, and were backcrossed at least 16 generations. Initial attempts to generate CaMKII KO mice were unsuccessful, as female CaMKII HT mice on a B6 background do not care for the pups, resulting in death of the entire litter. It was proposed by Dr. A. Silva that incorporation of a mixed background would produce CaMKII HT females that would nurse the pups; thus, in an effort to alleviate this problem, male CaMKII HT mice were paired with female WT B6129P3 hybrid mice (Jackson Laboratories) to produce F2 CaMKII HT mice on a mixed background. The male CaMKII HT mice and female hybrid CaMKII HT mice would not breed; thus no KO litters were produced. This led to the use of F2 CaMKII HT hybrids for our withdrawal studies.

Animals were 8-10 weeks of age, were group-housed in a 21°C humidity-controlled AAALAC-approved animal care facility with *ad libitum* access to food and water. Experiments were performed during the light cycle and were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

Drugs

(-)-Nicotine hydrogen tartrate salt, mecamylamine hydrochloride, verapamil hydrochloride, (±)Bay K8644, and KN93 were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Nimodipine was purchased from Research Biochemical International (RBI) (Natick, MA). KN92 was purchased from Seikagaku Corporation (Tokyo, Japan). Nicotine and mecamylamine were dissolved in physiological saline (0.9% sodium chloride) and injected s.c. at a volume of 10 ml/kg body weight. Verapamil, nimodipine, and (±)Bay K8644 were dissolved in a vehicle solution made of 5% ethyl alcohol, 5% emulphor oil, and 90% saline and administered by i.p. injection. KN92 and KN93 were diluted in saline and administered by i.c.v. injection. Doses are expressed as the free base of the drug.

I.c.v. surgery

I.c.v. surgery and injection were performed as described in Chapter 3. Briefly, a scalp incision was made to expose the bregma, and unilateral injection sites were prepared using a 26-gauge needle with a sleeve of PE tubing to control depth of the needle at a site 2 mm rostral and 2 mm lateral to the bregma at a depth of 2 mm. On the morning of testing, a volume of 5 µl using a 26-gauge needle with a sleeve of PE tubing was injected into the lateral ventricle and held in place for 20 seconds to ensure drug delivery.

Chronic nicotine administration protocol

Mice were implanted with Alzet osmotic mini pumps [model 2002 (14 days) or model 2004 (28 days) Durect Corporation, Cupertino, CA] filled with saline or (-)-nicotine (36 mg/kd/day) as described in Chapter 2. Due to ceiling effects with 36 mg/kg/day nicotine, in the (±)Bay K8644 assessment, mice used for this test were infused with 24 mg/kg/day nicotine.

Locomotor activity

Mice were injected with vehicle (i.p.) and immediately placed into individual photocell activity cages (28 x 16.5 cm; Omnitech, Columbus, OH) for a 30 minute habituation period. After habituation, mice were injected with vehicle or the assigned dose of nimodipine (1- 10 mg/kg, i.p.) and immediately returned to the locomotor cages. Interruptions of the photocell beams (two banks of eight cells each) were recorded for the next 30 min. Data are expressed as the number of photocell interruptions.

Nicotine withdrawal assessment

Withdrawal studies were conducted using the spontaneous withdrawal assessment as described in Chapter 3. In brief, for Ca^{2+} channel and pharmacological CaMKII studies, mini pumps were removed on day 14, and testing initiated on day 15, approximately 18-24 hours after mini pump removal. Mice were injected with vehicle, nimodipine (0.25 or 1 mg/kg, i.p.), verapamil (1 mg/kg, i.p.), or (±)Bay K8644 (0.25 and 0.5 mg/kg, i.p.) 15 minutes prior to initiation of testing or with KN93 or KN92 (0.0025- 0.01 µg/µl, i.c.v.) 5

minutes prior to testing. Mecamylamine-precipitated studies were conducted as described in Chapter 3. For KN93 precipitated studies, mice were injected i.c.v. and testing was initiated 5 minutes after the injection. The withdrawal testing sequence was conducted as described in previous chapters.

Nicotine CPA

Nicotine CPA testing was conducted as described in Chapter 2. Alterations to the procedure are described below.

Nimodipine assessment

On days 2 and 3 of CPA training, all mice received injections of saline in the morning. In the afternoon, mice received an injection of nimodipine (1 mg/kg, i.p.) or vehicle 15 minutes prior to mecamylamine injection. Mice were placed in the assigned chamber immediately after mecamyalmine injection for 30 minutes.

KN93 assessement

On day 4, test day, mice received i.c.v. injections of vehicle, KN93 (.01 μg/μl), or KN92 (.01 μg/μl) 5 minutes before being placed in the test chambers.

Statistical analysis

For all data, statistical analyses were performed using StatView ® (SAS, Cary, NC, USA). Data were analyzed with one-way ANOVAs with treatment as the between subject factor or two-way ANOVAs with treatment and genotype as between subject factors for the HT studies. Significant results were further analyzed using the Neuman-Keuls post-hoc test. *p* values of less than 0.05 were considered significant.

C. Results

Role of L-type VGCCs in physical and affective nicotine withdrawal

Before beginning our studies using L-type VGCC blockers, we wanted to determine the appropriate doses to use for our studies that would not have effects on locomotor activity. Preliminary data showed that nimodipine dose-dependently decreased locomotor activity in mice. Mice treated with 3 or 10 mg/kg nimodipine displayed a significant decrease in activity compared to vehicle treated mice (Fig. 20). There was no significant difference in activity between vehicle treated mice or mice treated with 1 or 2 mg/kg nimodipine. Based on this data, doses no higher than 2 mg/kg nimodipine were used for withdrawal studies.

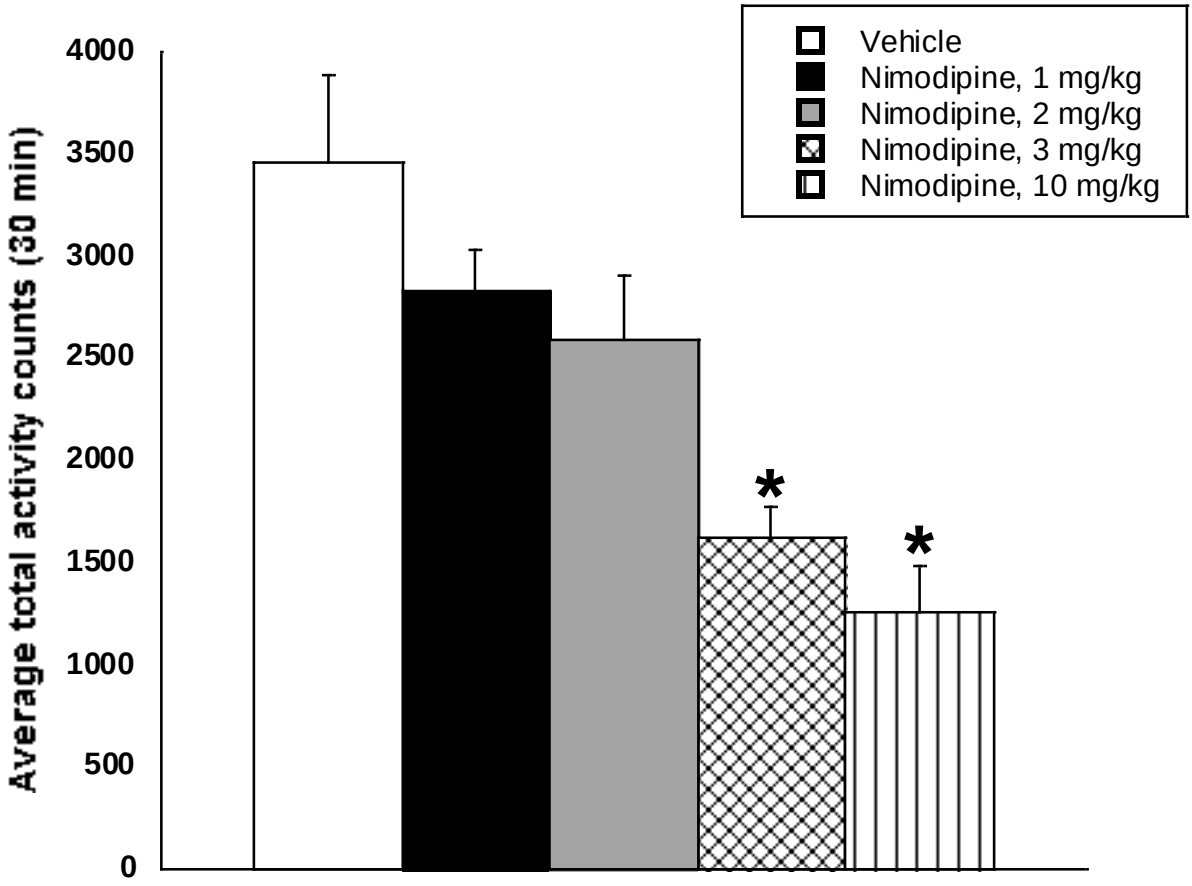


Figure 20. Nimodipine dose-dependently reduces locomotor activity in mice. Doses of 3 and 10 mg/kg nimodipine significantly reduced locomotor activity during the 30 minute test period; therefore, doses no higher than 2 mg/kg were used for our studies. Data are expressed as the number of photocell interruptions ± SEM for 6 mice per group. * denotes $p < 0.05$ vs. vehicle, 1 and 2 mg/kg nimodipine groups

As expected, nicotine-withdrawn mice pre-treated with vehicle showed a significant decrease in the amount of time spent on the open arms of the plus maze, indicating an anxiety-related response. Significant somatic signs and a significant decrease in hot plate latency compared to saline controls were also observed (Figure 21). Nimodipine dose-dependently reduced physical signs in nicotine-withdrawn mice. Mice treated with 1 mg/kg nimodipine, but not 0.25 mg/kg, showed a significant decrease in total somatic signs and a significant increase in hot plate latency, indicating a loss of the hyperalgesia response (Fig. 21B and 21C). There was no significant difference in time spent on the open arms of the plus maze in nicotine-withdrawn mice treated with nimodipine compared to vehicle, suggesting that nimodipine had no effect on anxiety-related behavior (Fig. 21A). Verapamil, an L-type VGCC blocker structurally different from nimodipine, had effects similar to nimodipine in nicotine-withdrawn mice. Verapamil (1 mg/kg, i.p.) significantly reduced somatic signs and significantly increased hot plate latency in nicotine-withdrawn mice; however, verapamil had no effect on anxiety-related behavior as measured by the plus maze (Fig. 21). The Ca^{2+} channel blocker doses had no significant effect in saline infused mice, indicating that, by themselves, the doses used were not behaviorally active (Fig. 21).

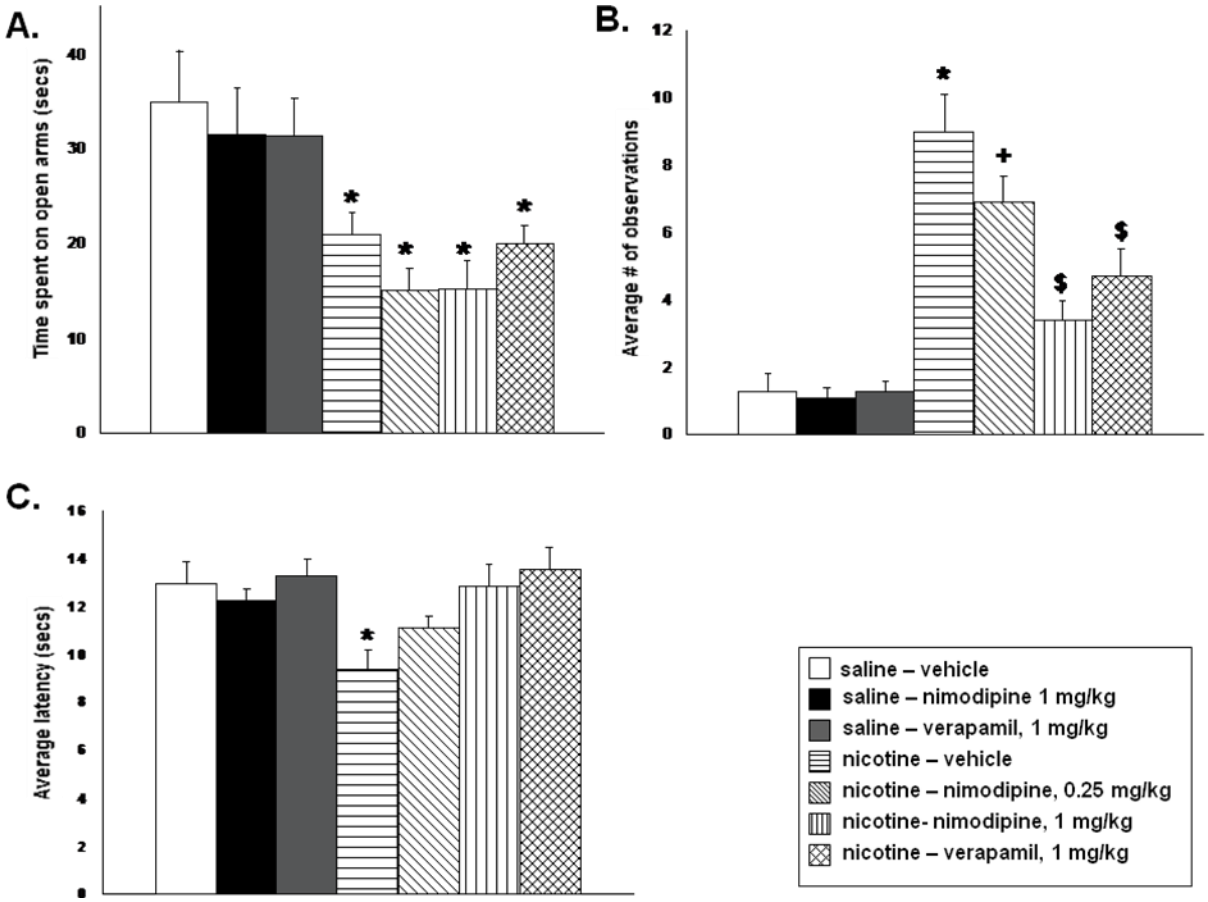


Figure 21. L-type VGCCs are involved in physical, but not affective withdrawal. Nimodipine (1 mg/kg, i.p.) and verapamil (1 mg/kg, i.p.) B. attenuated somatic withdrawal signs and C. attenuated the hyperalgesia response as measured by a significant increase in hot plate latency; however, A. had no effect on anxiety-related behavior as measured by the amount of time spent on the open arms of the plus maze. Each point represents the mean ± SEM 8 mice per group. * denotes p < 0.05 vs. saline controls and vs. nicotine (nic) –nimodipine (nim)/verapamil (ver) for the hot plate assessment. + denotes p < 0.05 vs. nic –vehicle (veh). $ denotes p < 0.05 vs. saline controls, nic- nim, 1 mg/kg and nic-min 0.25 mg/kg

L-type VGCC blockers have been shown to inhibit α3* and α7* nAChR-mediated currents and downstream signaling *in vitro* at doses typically used to block L-type VGCC (Wheeler et al., 2006); therefore, mice were treated with the L-type VGCC activator, (±)Bay K8644, after nicotine withdrawal to complement results observed with L-type VGCC blockers. Because (±)Bay K8644 has significant effects on locomotor activity as measured by the number of arm crosses on the plus maze (saline-veh: 4 ± 0.27 crosses vs. saline-BayK: 11 ± 1.67 crosses), animals were not evaluated in the plus maze, as changes in locomotor activity can confound results in this test. (±)Bay K8644 dose-dependently enhanced somatic signs and the hyperalgesia response in nicotine-withdrawn mice. Compared to vehicle treated nicotine-withdrawn mice, mice treated with 0.5 mg/kg (±)Bay K8644, but not 0.25 mg/kg, exhibited significantly more somatic signs (Fig. 22A) and a significant decrease in hot plate latency (Fig. 22B), indicating an enhanced response in both behavioral tests.

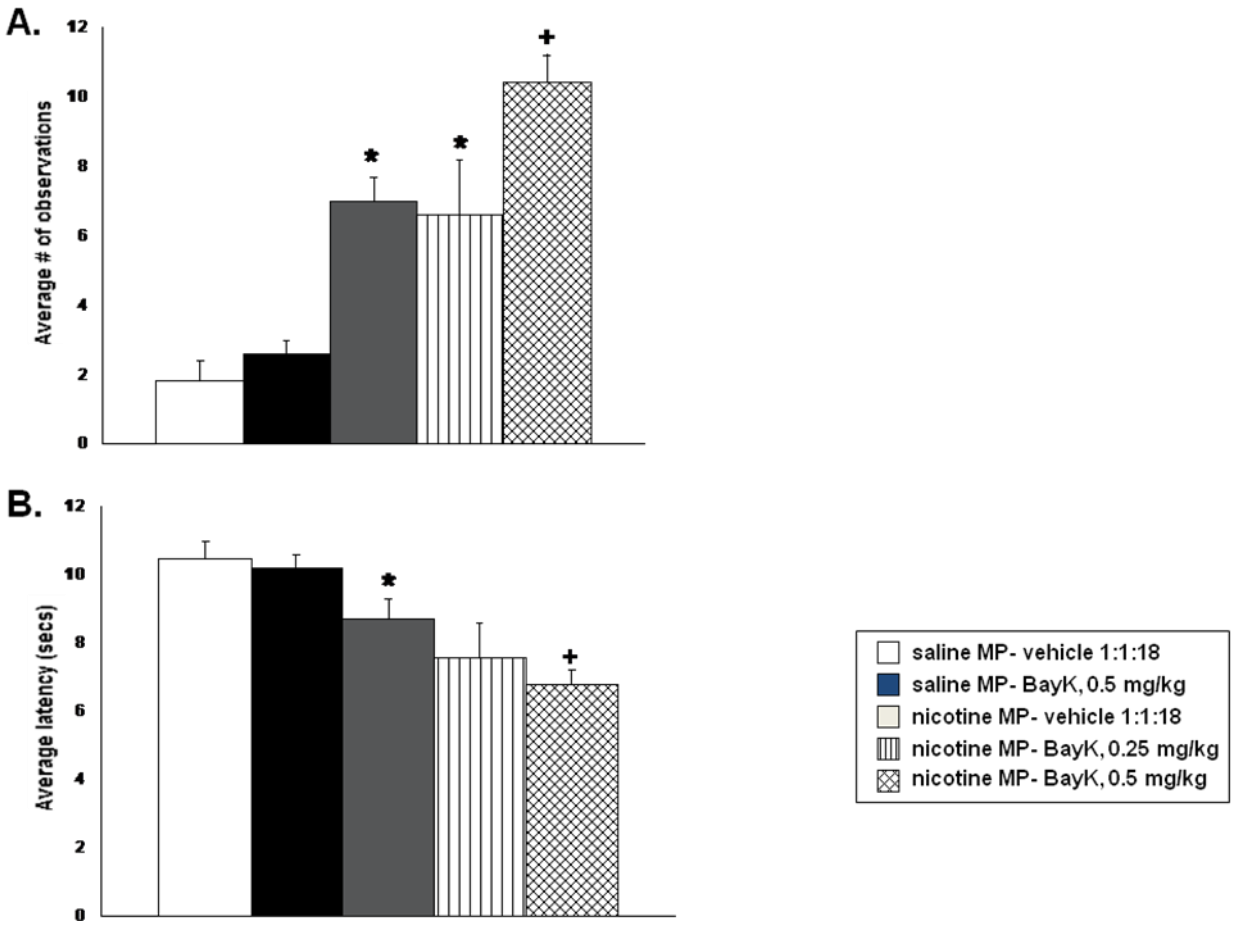


Figure 22. (±)Bay K8644 dose-dependently enhances physical withdrawal signs. Nicotine-withdrawn mice treated with (±)Bay K8644 (BayK, 0.5 mg.kg, i.p.) exhibited A. a significant increase in somatic signs and B. and a significant decrease in hot plate latency when compared to nicotine-withdrawn vehicle (veh) treated mice. Each point represents the mean ± SEM of 8 mice per group. * denotes $p < 0.05$ vs. saline controls. + denotes $p < 0.05$ vs. nicotine- veh mice

Evaluation of L-type VGCCs using the CPA model

Results suggested that L-type VGCCs are involved in physical, but not affective nicotine withdrawal. To further assess this effect, the aversion associated with nicotine withdrawal was measured using the CPA model. Mice were pre-treated with nimodipine (1 mg/kg, i.p.), 15 minutes prior to mecamylamine injection during conditioning. Mecamylamine (3.5 mg/kg, s.c.) precipitated significant CPA in chronic nicotine infused mice compared to saline infused mice (Fig. 23). Pre-treatment with nimodipine had no effect on development, as mice pre-treated with nimodipine expressed significant aversion compared to saline counterparts at a dose that did not produce significant behavioral effects in saline-infused mice (Fig. 23).

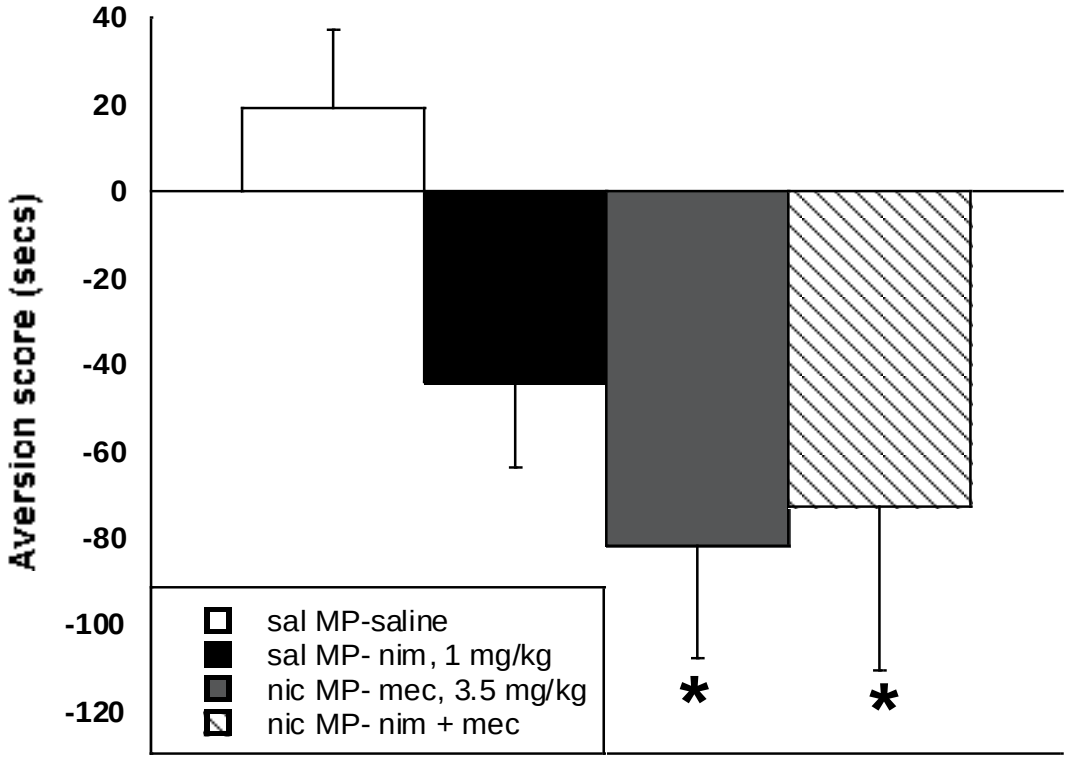


Figure 23. L-type VGCCs are not involved in development of mecamylamine-precipitated CPA. Mecamylamine precipitates aversion in nicotine infused mice. Pre-treatment with nimodipine had no effect on the development of mecamylamine-precipitated aversion. Each point represents the mean ± SEM of 12 mice per group. * denotes $p < 0.05$ vs. saline group.

Role of CaMKII in physical and affective nicotine withdrawal

To assess the role of CaMKII in nicotine withdrawal, we began with a pharmacological assessment using KN-93, a CaMKII inhibitor. KN93 is potent, cell-permeable, and can be dissolved in water instead of DMSO, thus avoiding side effects of this vehicle (Gao et al., 2006). As a control, we also used the inactive analog of KN93, KN92, which does not inhibit kinase activity. KN93, however, inhibits other kinases, such as CaMKIV (Enslen et al., 1994); therefore, to complement our pharmacological assessment, we tested transgenic mice in our withdrawal model. While the initial goal was to obtain CaMKII KO mice for our studies, female HT mice either did not care for the pups, so we could not maintain live litters, or the mice did not breed; therefore, we used CaMKII HT mice to complement our pharmacological studies.

Nicotine-withdrawn mice were treated with vehicle or a range of KN93 doses prior to initiation of testing. KN93 dose-dependently reduced somatic signs in nicotine-withdrawn mice (Fig. 24B). Mice treated with 0.0025 μg/μl showed a significant reduction in the total number of somatic signs compared to nicotine-withdrawn mice treated with vehicle, while somatic signs were attenuated in nicotine-withdrawn mice treated with 0.005 and 0.01 μg/μl KN93 (Fig. 24B). In the hyperalgesia assessment, nicotine-withdrawn vehicle treated mice displayed a significant reduction in hot plate latency compared to saline infused animals (Fig. 24C). Treatment with KN93 had no effect on hot plate latency at any dose tested (Fig. 24C). Interestingly, there was a significant reduction in the amount of time spent on the open arms of the plus maze in KN93 treated nicotine-withdrawn mice

compared to vehicle treated nicotine withdrawn mice. The highest dose of KN93 (0.01 μg/μl) reduced the amount of time spent on the open arms of the plus maze compared to vehicle treated nicotine-withdrawn mice, indicating an enhanced anxiety-related response after KN93 treatment (Fig. 24A). There was no significant difference in the number of arm crosses between groups, suggesting that the effect was not attributed to differences in locomotor activity (Table 4). Nicotine-withdrawn mice treated with the inactive analog, KN92 (0.01 μg/μl, i.c.v.) did not differ from their vehicle treated counterparts in any behavioral test, and the highest dose of KN93 (0.01 μg/μl) was not behaviorally active in saline infused mice.

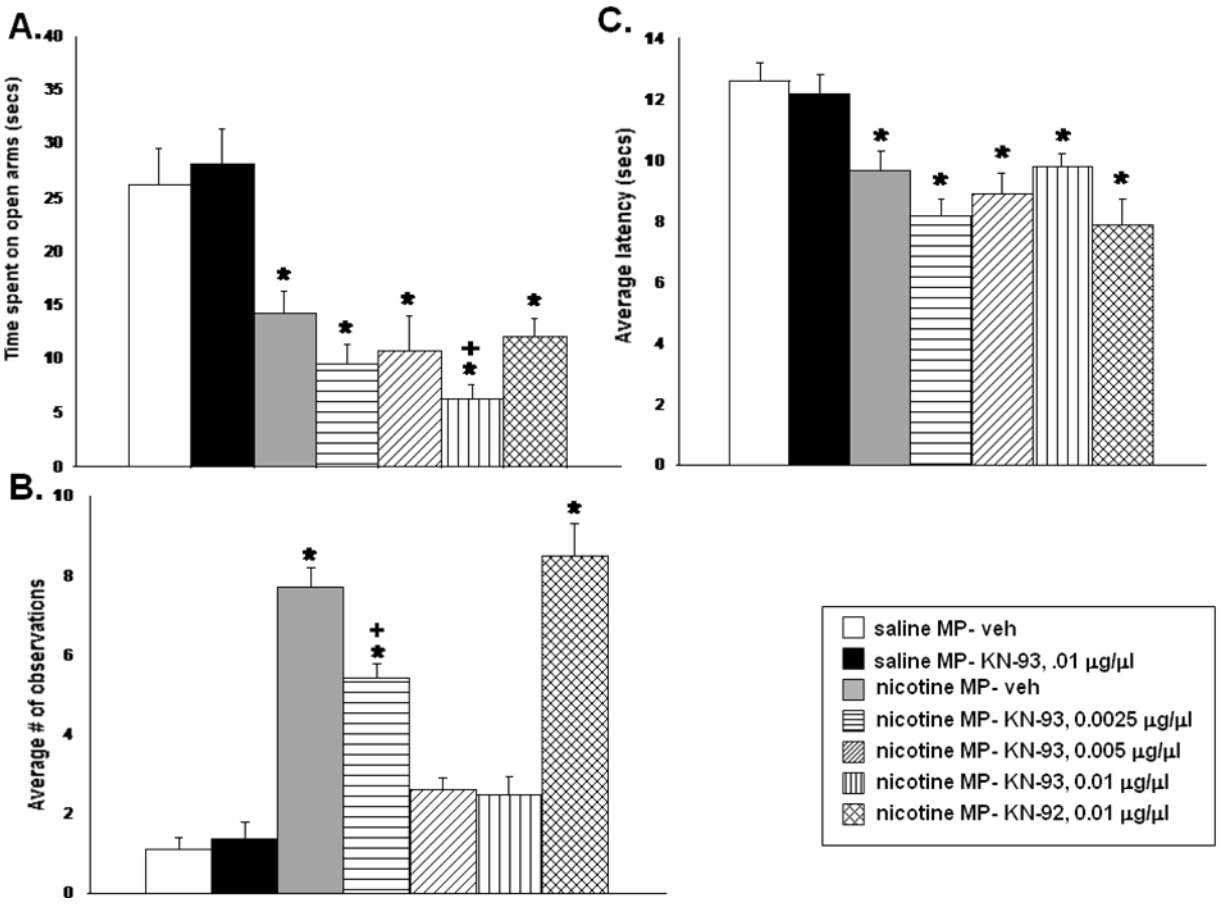


Figure 24. KN93 attenuates somatic signs, but enhances anxiety-related behavior. Nicotine-withdrawn mice were treated with various doses of KN93. Results show A. KN93 dose-dependently reduces time spent on the open arms of the plus maze, suggesting an enhanced anxiety-related response, B. a dose-dependent attenuation of somatic signs after KN93 treatment, but C. no effect on the hyperalgesia response after any KN93 dose tested. The highest dose of KN93 (0.01 µg/µl) did not produce effects in saline treated mice, indicating that the dose is not behaviorally active by itself. The inactive analog KN92 also did not produce behavioral effects in any withdrawal assessment. Each point represents the mean ± SEM of 10 mice per group. * denotes $p < 0.05$ vs. saline groups. + denotes $p < 0.05$ vs. nicotine-vehicle group.

Table 4. Average number of arm crosses in the KN93 plus maze assessment

Nicotine-withdrawn mice were treated with vehicle (veh), KN93, or the inactive analog KN92, and the total number of crosses between open and closed arms of the plus-maze test was counted. Numbers are presented as the total average number of arm crosses ± SEM for 8 mice per group.

Treatment group	Average number of arm crosses
Saline MP- veh	2.4 ± 0.3
Saline MP- KN93, 0.01 μg/μl	2.5 ± 0.6
Nicotine MP- veh	2 ± 0.5
Nicotine MP- KN93, 0.0025 μg/μl	2.2 ± 0.6
Nicotine MP- KN93, 0.005 μg/μl	2 ± 0.6
Nicotine MP- KN93, 0.01 μg/μl	2 ± 0.3
Nicotine MP- KN92, 0.01 μg/μl	2.2 ± 0.4

Results suggested that CaMKII has opposite roles in nicotine withdrawal. While there was an attenuation of somatic signs, we observed an enhancement of the anxiety-related response. To further examine this response, we tested the effect of KN93 in a precipitated nicotine withdrawal model. Mini pumps were not removed on day 14, and withdrawal signs were measured the morning of day 15 following administration of saline, mecamylamine, or KN93. Pretreatment with mecamylamine (2mg/kg, s.c.) or KN93 (0.01 μg/μl, i.c.v.) precipitated a significant decrease in the open arms of the plus maze in chronic nicotine infused mice, indicating an anxiety-related response (Fig. 25A). While mecamylamine also precipitated a significant increase in somatic signs and a significant decrease in hot plate latency in nicotine-infused mice, KN93 failed to precipitate physical nicotine withdrawal signs (Fig. 25B and 25C).

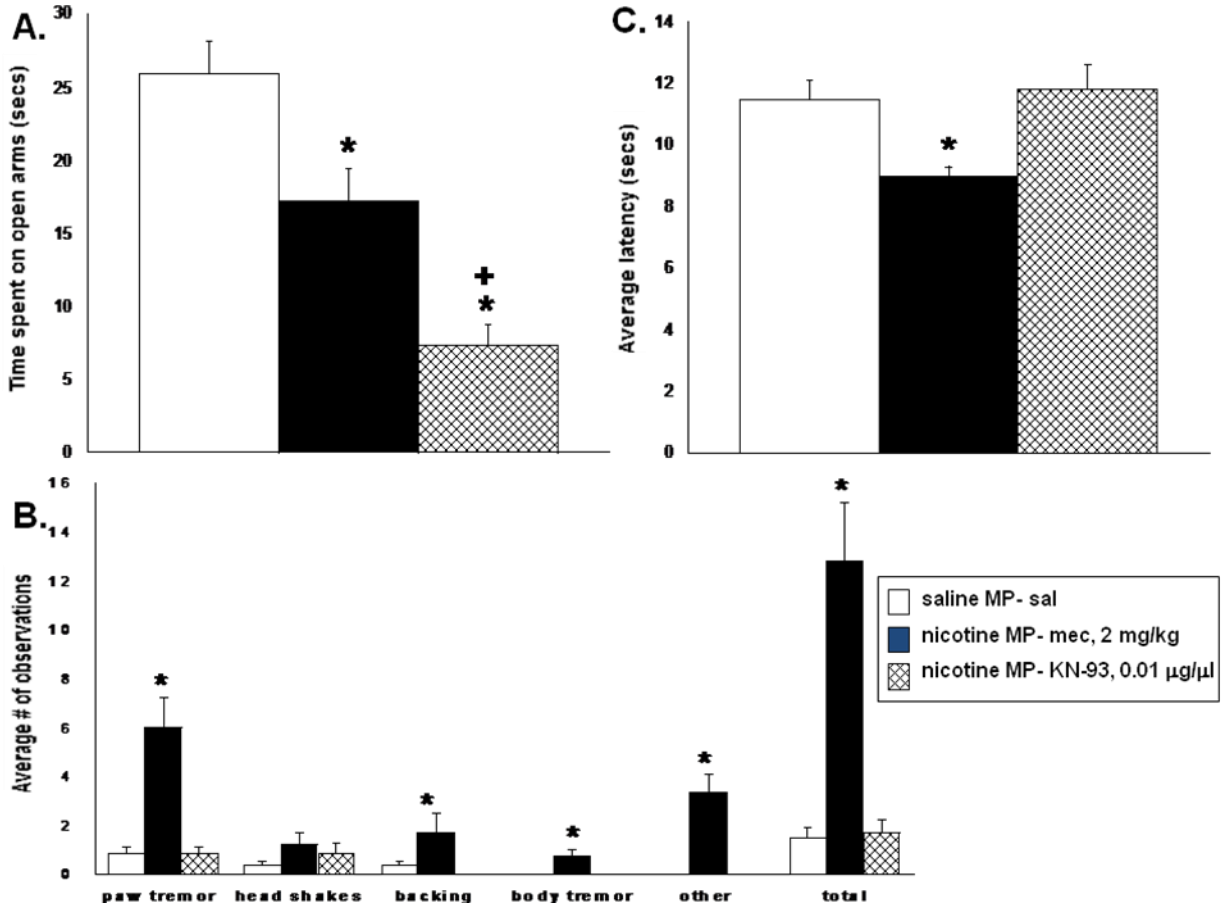


Figure 25. KN93 precipitates anxiety-related behavior, but not physical withdrawal signs. Withdrawal was precipitated with KN93 or mecamylamine in chronic nicotine infused mice. Mice show A. a significant decrease in the amount of time spent on the open arms of the plus maze, indicating an anxiety-related response after KN93 and mecamylamine treatment. B. KN93 did not precipitate somatic signs or C. a hyperalgesia response in chronic nicotine infused mice as seen after mecamylamine injection. Each point represents the mean ± SEM of 8 mice per group. * denotes p< 0.05 vs. saline group. + denotes p< 0.05 vs. mecamylamine group.

While KN93 is a selective CaMKII inhibitor, it may also inhibit other CaMKs, such as CaMKIV (Enslen et al., 1994); therefore, to complement our pharmacological approach, we measured withdrawal signs in nicotine infused CaMKII HT mice after mecamylamine injection. Mecamylamine precipitated an anxiety-related response in nicotine infused CaMKII WT mice (Fig. 26A). Interestingly, saline infused CaMKII HT mice had significantly higher plus maze baseline activity than their WT counterparts, and mecamylamine did not precipitate an anxiety-related response in nicotine infused HTs (Fig. 26A). There was also no significant difference between nicotine infused HT and WT mice in either physical measure, as mecamylamine precipitated a significant increase in somatic signs and decrease in hot plate latency in both nicotine infused HT and WT mice (Fig. 26B and 26C).

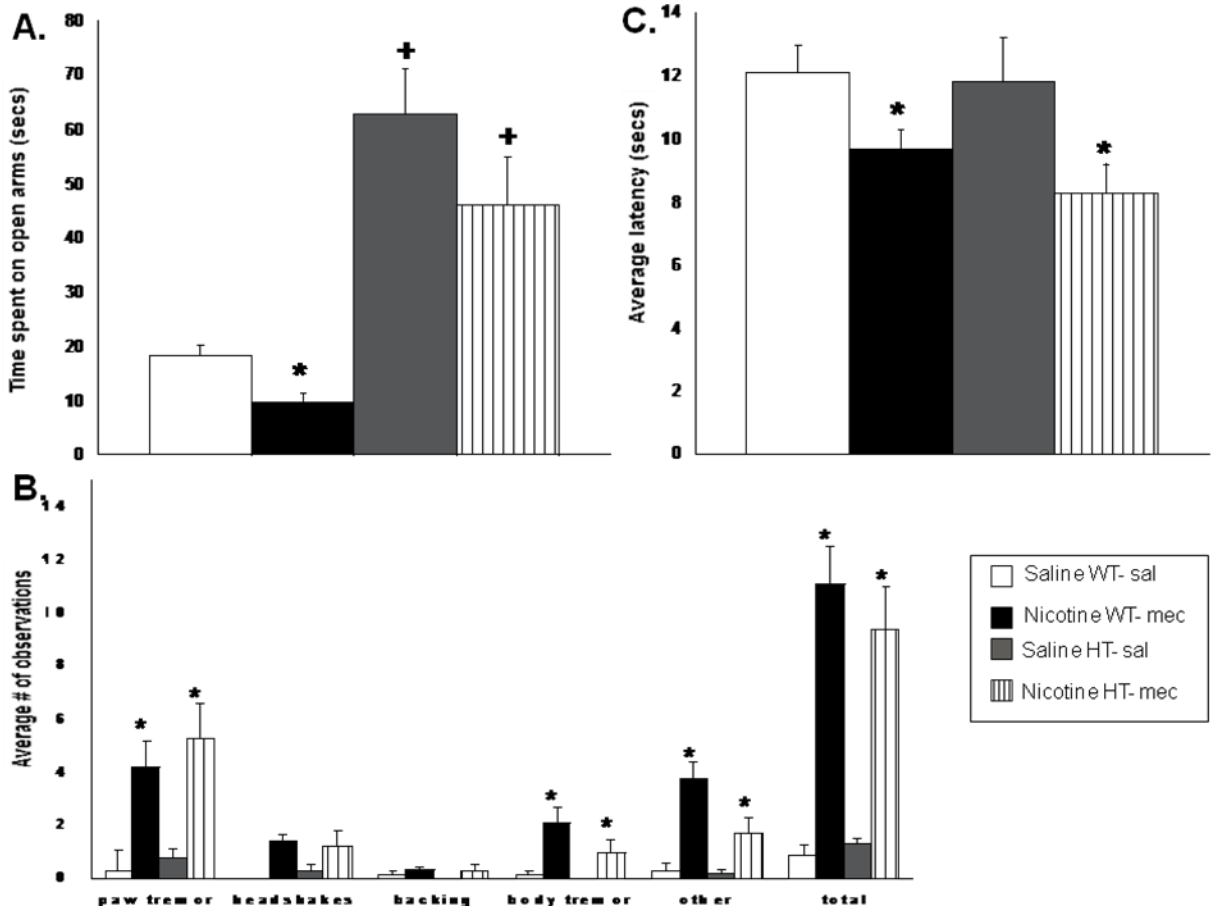


Figure 26. Evaluation of the role of CaMKII in nicotine withdrawal using CaMKII HT mice. A. Saline infused CaMKII HT mice spend significantly more time on the open arms compared to WT mice, indicating higher baseline levels on the plus maze, while nicotine infused CaMKII HTs did not display an anxiety response. B. Nicotine infused CaMKII HTs and WTs both exhibit significant somatic signs after mecamylamine injection compared to saline counterparts. C. Mecamylamine precipitates a significant hyperalgesia response in CaMKII HT and WT mice. Each point represents the mean ± SEM of 10 mice per group. * denotes $p < 0.05$ vs. saline groups. + denotes $p < 0.05$ vs. WT mice

Role of CaMKII in affective withdrawal using the CPA model

In an attempt to generate CaMKII KO mice, male CaMKII HT mice were bred with female WT B6129P3 hybrid mice to produce F2 CaMKII HT mice on a mixed background for our studies; therefore, we evaluated development of CPA in the hybrid background strain. Results show that both male and female B6129 mice develop significant mecamylamine-precipitated aversion in the CPA model (Fig. 27). For pharmacological CaMKII studies assessing expression of CPA, vehicle, KN93, or KN92 were administered to mice on test day, 5 minutes prior to entering the chamber. Mecamylamine (3.5 mg/kg, s.c.) precipitated aversion in nicotine infused mice treated with vehicle i.c.v. (Fig. 28A). KN93 (0.01 μg/μl, i.c.v.), but not KN92 (0.01 μg/μl, i.c.v.) showed a strong trend toward enhanced expression of mecamylamine-precipitated CPA that did not reach statistical significance ($p= 0.06$). The dose of KN93 used did not produce CPA in saline infused mice. Activity counts showed no significant between group differences in activity on test day (Table 5), suggesting that results were not attributed to differences in chamber activity. To complement the pharmacological approach, CaMKII HT mice were evaluated using the CPA model. Mecamylamine precipitated significant aversion in CaMKII WT and HT mice (Fig. 28B). There was no significant difference in activity count between groups (Table 5).

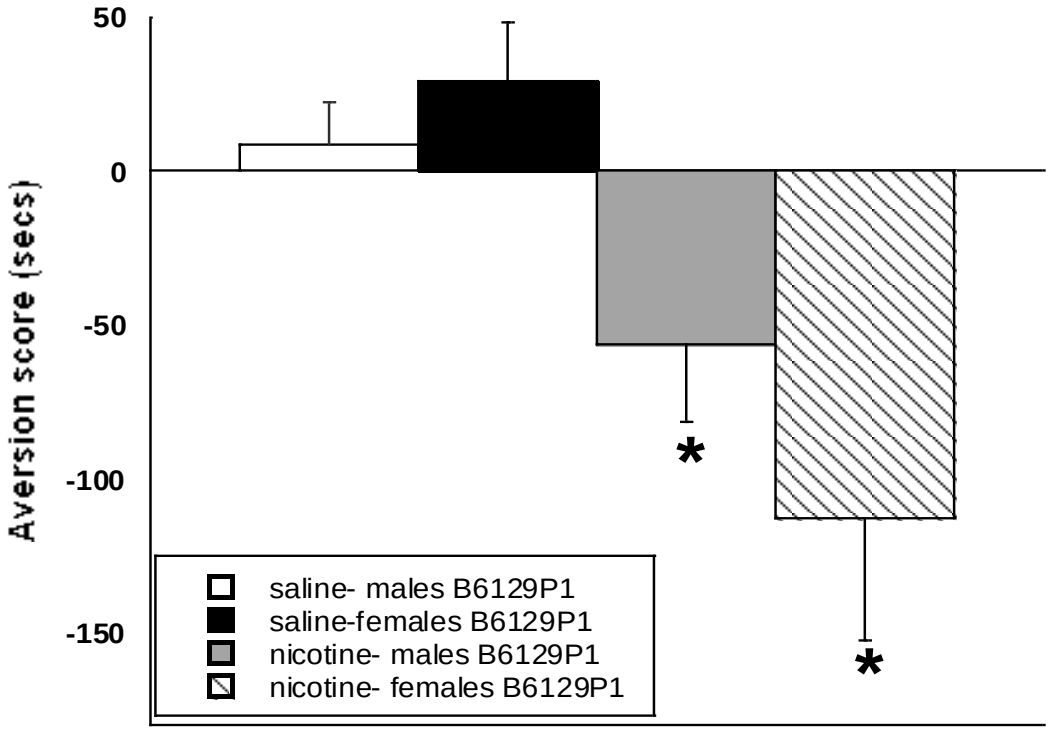


Figure 27. Assessment of male and female B6129P3 hybrid mice in the development of aversion in the CPA model. Nicotine infused male and female B6129P3 hybrid mice develop aversion after mecamylamine conditioning in the CPA model. Each point represents ± S.E.M. of 12 mice per group. * denotes $p<0.05$ vs. the corresponding saline group

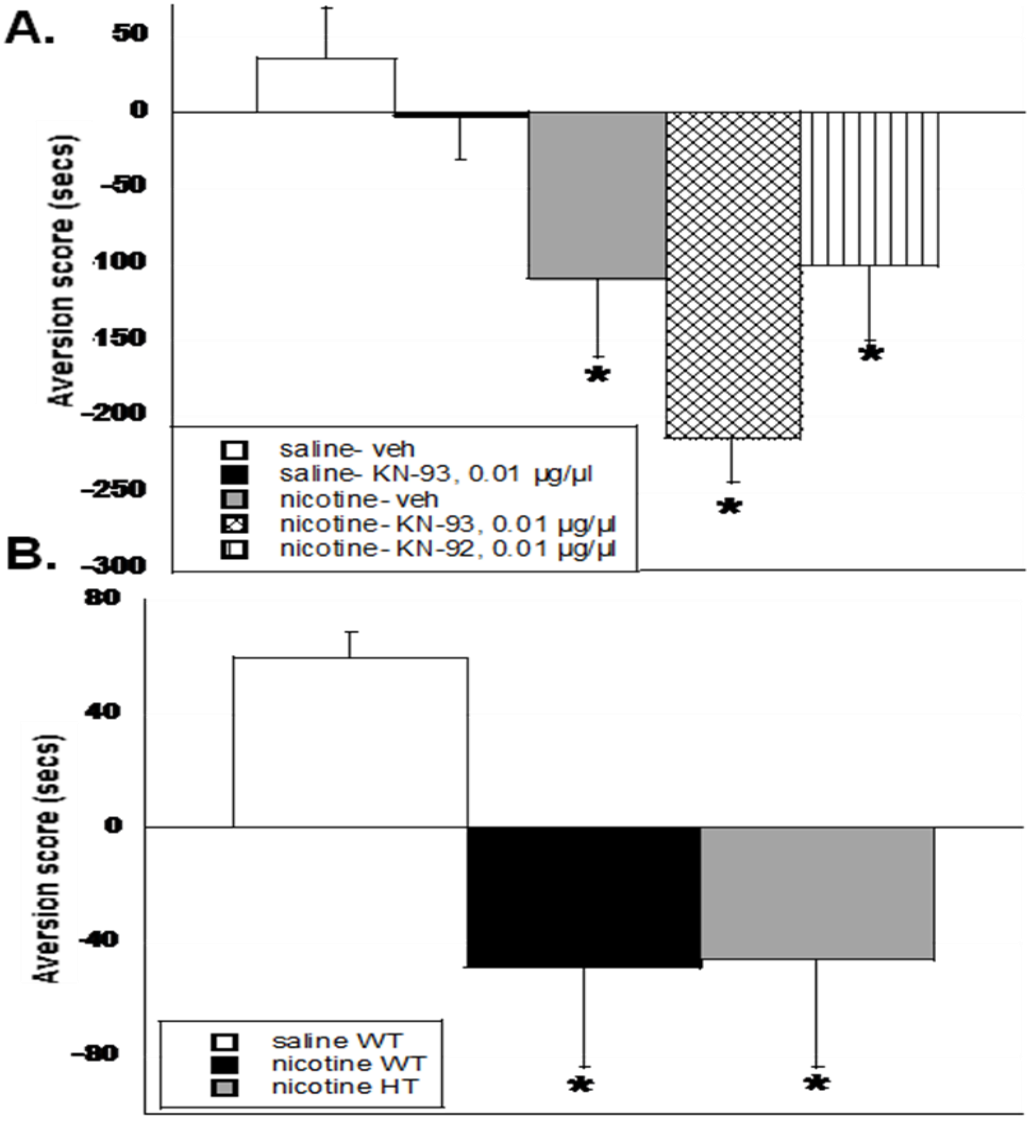


Figure 28. Inhibition of CaMKII enhances the aversion associated with nicotine withdrawal. A. Nicotine infused mice treated with KN93 on test day express a strong trend that did not reach statistical significance toward enhanced CPA compared to nicotine-infused mice treated with vehicle (veh) or KN92 (p=0.06). B. Mecamylamine precipitates aversion in CaMKII WT and HT mice. There was no significant difference in the level of aversion between WT and HT mice. Each point represents the mean ± SEM of 12 mice per group. * denotes p < 0. 05 vs. saline groups.

Table 5. Average activity counts in the CPA model for the CaMKII assessment

Mice were treated with vehicle (veh) or KN93 (0.01μg/μl) i.c.v. for the pharmacological assessment on test day. CaMKII HT and WT mice were also evaluated in the CPA model. Activity counts were taken for each compartment in the CPA chamber. Numbers represent the total activity counts in drug-paired compartment on test day for each group and are presented as the average activity count on test day (post-conditioning day) ± S.E.M. for 12 mice.

Treatment groups	**Average activity counts- drug-paired compartment**
Saline MP- veh	438 ± 78.1
Saline MP-KN93	369.9 ± 34.2
Nicotine MP- veh	437 ± 97.8
Nicotine MP- KN93	455.8 ± 185.1
Nicotine MP- KN92	492.9 ± 92.9
Saline CaMKII WT	624.1 ± 45
Nicotine CaMKII WT	671 ± 50.5
Nicotine CaMKII HT	692.9 ± 36.4

D. Discussion

The main goal of this study was to investigate the role of L-type VGCCs and CaMKII in nicotine withdrawal. The overall results suggest that Ca^{2+}-dependent mechanisms are involved in physical and affective nicotine withdrawal. L-type VGCC blockers attenuated nicotine withdrawal somatic signs and the hyperalgesia response, and (±)BayK 8644, an L-type VGCC activator, enhanced these signs. There was no effect in the plus maze or CPA model, suggesting a role for L-type VGCCs in physical, but not affective withdrawal. The results of the CaMKII assessment were not as clear. The pharmacologically CaMKII assessment revealed that KN93 dose-dependently attenuated somatic withdrawal signs, but enhanced the affective response, suggesting differential roles for CaMKII in somatic and affective nicotine withdrawal. Surprisingly, the transgenic data using CaMKII HT mice suggests a role opposite that of the pharmacological assessment.

The L-type VGCC blockers, nimodipine and verapamil, attenuated the expression of nicotine withdrawal-induced somatic signs and the hyperalgesia response, but had no effect on the anxiety-response or CPA, suggesting a role for L-type VGCCs in physical, but not affective nicotine withdrawal. These results are consistent with a previous study by Biala and Weglinska (2005), which treated mice with various L-type VGCC blockers after mecamylamine-precipitated withdrawal. Despite the complementary results, in our study, we show that the doses used in the Biala study (5 and 10 mg/kg of each L-type VGCC blocker), significantly depress locomotor activity in mice, which could impact data interpretation in a withdrawal study. Further, the Biala study used the mecamylamine-

precipitated model to evaluate expression of nicotine withdrawal. It is unknown whether the combination of mecamylamine and L-type VGCCs produces a drug interaction that influences the results; thus, to avoid such an interaction, the current study utilized a spontaneous nicotine withdrawal model and L-type VGCC blocker doses that do not impact locomotor activity. L-type VGCC blockers have been shown to inhibit α3* and α7* nAChR-mediated currents and downstream signaling *in vitro* at doses typically used to block L-type VGCC (Wheeler et al., 2006); therefore, mice were also treated with the L-type VGCC activator, (±)Bay K8644. Nicotine-withdrawn mice treated with (±)Bay K showed enhanced somatic signs and an enhanced hyperalgesia response. Taken together, these results suggest an important role for L-type VGCCs in physical, but not affective nicotine withdrawal. The results may also highlight a potential role for indirect sources of nicotine-induced Ca^{2+} influx. Upon nicotine binding, there is a direct Ca^{2+} influx through the Ca^{2+} permeable nAChR. The resulting increase in intracellular Ca^{2+} leads to an indirect Ca^{2+} influx through VGCC as a result of membrane depolarization following nAChR activation. Because physical signs are altered by L-type VGCC pharmacological agents, this would suggest an important role for a mechanism of Ca^{2+} influx that occurs as a result of nAChR activation.

The intracellular rise in Ca^{2+} through nAChRs leads to activation of CaMKII. Our data in chapter 6 shows that acute nicotine induces increases in CaMKII activity in the VTA and NAc. Additionally, previous data reports increases in CaMKII function in PC12 cells (MacNicol and Schulman, 1992), and in the spinal cord membrane after nicotine

treatment (Damaj, 2000). Our pharmacological data showed that the CaMKII inhibitor KN93 dose-dependently attenuated somatic signs, but enhanced the expression of the anxiety-related response. There was also a trend toward enhancement of CPA expression after KN93 treatment on test day. It is possible, in the CPA model, that a floor effect prevented the aversive response after KN93 treatment from reaching significance. Using a lower dose of nicotine (24 mg/kg/day instead of 36 mg/kg/day) in the CPA model for future studies may prevent this floor effect, and provide clearer results. Overall, these results imply that CaMKII has differential roles in somatic and affective nicotine withdrawal. While KN93 enhanced the anxiety-related response, suggesting that CaMKII activity is decreased after nicotine withdrawal, the antagonist attenuated somatic signs, suggesting that CaMKII is increased after nicotine withdrawal.

In Chapters 3 and 4, we found that somatic and affective nicotine withdrawal signs are mediated by different subtypes; thus, it is possible that the opposing roles of CaMKII in somatic and affective withdrawal could reflect the involvement of different nAChR subtypes. It may also be possible that the effect of KN93 is attributed to blockade of nAChRs. KN93 has been shown to reversibly block α3* and α7* nAChR-mediated responses *in vitro* (Liu and Berg, 1999). Our studies in Chapter 4 implicate the α3 nAChR subunit in somatic nicotine withdrawal; thus, it may be possible that the attenuation of somatic signs by KN-93 is attributed to blockade of α3-containing nAChRs. It is currently unknown if KN93 blocks α4β2* nAChRs, which was shown in Chapter 3 to be necessary for affective nicotine withdrawal. An electrophysiological evaluation that is beyond the

scope of our studies would be necessary to examine α4β2* nAChR responses after KN93 treatment. Additionally, KN-93 inhibits L-type VGCCs *in vitro* (Gao et al., 2006). Although KN-93 had no effect on the hyperalgesia response, as was observed with L-type VGCC blockers, we cannot rule out the possibility that the effects on somatic signs may involve inhibition of this target.

While the pharmacological results suggested a role for CaMKII in somatic and affective nicotine withdrawal, the assessment using CaMKII HT mice produced different results. There was no significant difference in somatic signs or hyperalgesia response between nicotine and saline infused CaMKII HT and WT mice after mecamylamine treatment, suggesting that CaMKII is not involved in the development of physical nicotine withdrawal signs. Interestingly, there was a loss of the anxiety-related response in nicotine infused CaMKII HT mice, as there was no decrease in time spent on the open arms after mecamylamine treatment, and no difference between CaMKII HT and WT mice in development of a significant CPA. The difference in affective measures may be explained by the fact that the anxiety-related behavior and aversive responses involve two different brain regions. Although these results suggest that CaMKII is involved in the nicotine withdrawal-induced anxiety-related response, the transgenic mouse data suggests CaMKII function is increased after nicotine withdrawal, which is the opposite of what was observed in the pharmacological KN93 assessment. One possible explanation for the discrepancy between our pharmacological and transgenic data is the lack of selectivity of KN93. KN93 blocks CaMKIV activity at an IC_{50} of 3 μM, a concentration only 4-fold higher than the

IC_{50} value for CaMKII (0.8 μM) (Hook and Means, 2001). It is therefore possible that the observed effects are attributed to blockade of CaMKIV activity rather than CaMKII. However, based on diffusion studies by Matta et al. (1995), the highest dose of KN93 (0.01μg/μl) would correspond to a tissue concentration of ~ 0.7 μM, which is below the IC_{50} value sufficient to block CaMKIV activity. Another possibility is that the comparison of CaMKII HT mice to the pharmacological data is unclear. The CaMKII HT mice possess 50% of the CaMKII enzyme, and it is unknown whether this enzyme level is sufficient for expression of the alterations in nicotine withdrawal observed after KN93 treatment. It is possible that total blockade of CaMKII activity is necessary for expression of attenuated somatic signs and an enhanced anxiety-related response. The CaMKII HT mice were also bred on a mixed genetic background containing B6 and B6129 hybrids. Many studies support an important role for a genetic component to nicotine dependence; thus, we cannot rule out the possibility that the mixed genetic background contributed to the observed phenotype in CaMKII HT mice. Our breeding attempts found that the CaMKII HT mothers maintained on a B6 background breed and give birth to live litters, but do not care for the pups; thus we could not obtain CaMKII KO mice for our studies. For future studies, rather than incorporating a mixed background strain, we will attempt to foster the pups from HTs maintained on a B6 background with WT mice to obtain KOs.

The overall results of this study suggest that different Ca^{2+}-dependent mechanisms are involved in physical and affective nicotine withdrawal. L-type VGCCs mediate somatic signs and hyperalgesia, suggesting the importance of the indirect Ca^{2+} influx in expression

of physical nicotine withdrawal. The role of CaMKII in nicotine withdrawal behaviors is more complex. Pharmacological data supports a role for CaMKII in having opposing roles in nicotine withdrawal, as an antagonist attenuates somatic signs, yet enhances affective signs; however, the transgenic assessment did not elucidate a role for CaMKII in nicotine withdrawal. While the current study, taken together with previous biochemical studies, does implicate a role for CaMKII in nicotine dependence, further work is necessary to examine the behavioral specifics of CaMKII in nicotine withdrawal. The next chapter focuses on exploring the relationship between changes in the function of CaMKII and synapsin I, a CaMKII substrate necessary for neurotransmitter release after nicotine withdrawal, and *in vivo* nicotine withdrawal induced behavioral alterations.

IN VITRO CHARACTERIZATION OF MOLECULAR MECHANISMS OF NICOTINE WITHDRAWAL: A ROLE FOR CAMKII AND SYNAPSIN I

A. Introduction

In the previous chapter, we found that Ca^{2+} -dependent mechanisms, specifically L-type VGCC and CaMKII, are involved in nicotine withdrawal behaviors. While L-type VGCC blockers attenuated physical withdrawal signs, L-type VGCC activators had the opposite effect. Further, CaMKII inhibitors attenuated somatic signs, but enhanced anxiety-related behavior and aversion. Since these studies showed that these Ca^{2+}-dependent mechanisms are relevant to nicotine withdrawal behaviors, we wanted to examine the relationship between the altered behavioral response *in vivo*, and the *in vitro* Ca^{2+}-dependent molecular mechanisms involved in nicotine withdrawal. We focused our studies on CaMKII and its presynaptic substrate, synapsin I.

Synapsin I is a presynaptic vesicle-associated protein that is essential for neurotransmitter release. It is phosphorylated and activated by various protein kinases, including PKA, MAPK, cyclin-dependent protein kinases (cdks), and CaMKII, which phosphorylates the protein at Ser-566 and Ser-603 (De Camilli et al., 1990; Hilfiker et al., 1999). Phosphorlyation of synapsin I by CaMKII in particular causes the protein to dissociate from the synaptic vesicle and allows the vesicle to move to and fuse with the plasma membrane for neurotransmitter release (De Camilli et al., 1990; Greengard et al., 1993). Indeed, experimental data indicates an important role of synapsin I phosphorylation by CaMKII in the regulation of neurotransmitter release. It was shown that introduction of activated CaMKII into rat synaptosomes induces release of glutamate and noradrenaline,

and this release is reduced by CaMKII inhibitors (Nichols et al., 1990). Further, using giant squid synapses, it was shown that CaMKII injected presynaptically facilitates neurotransmitter release by phosphorylation of synapsin I (Llinás et al., 1991). Synapsin I has also been suggested as a candidate for mediating molecular processes associated with learning and memory, such as LTP. There was an increase in synapsin I concentration in dentate gyrus slices after induction of LTP (Lynch et al., 1994). This effect was also observed *in vivo*, where LTP was induced in the dentate gyrus of rats by increasing neuronal activity, and increases in synapsin I mRNA were detected (Hicks et al., 1997).

This is of particular relevance since it has been suggested that addiction and learning and memory share common molecular substrates and pathways, such as intracellular signaling cascades that lead to activation of the transcription factor CREB (Nestler, 2001b; Nestler, 2002). In fact, like CaMKII, synapsin I activity and expression are altered by various drugs of abuse. Synapsin I mRNA was increased in the LC, amygdala, spinal cord, and pontine central gray area after chronic morphine treatment in rats, suggesting that increases in synapsin I in these particular brain areas, which are important for opiate action, may be part of the molecular mechanisms underlying opiate tolerance and withdrawal (Matus-Leibovitch et al., 1995). Further, increased synapsin I phosphorylation and subsequent DA release were noted after amphetamine sensitization in rats, and after chronic amphetamine treatment in rat striatal synaptosomes (Iwata et al., 1996; Iwata et al., 1997a; Iwata et al., 1997b). Taken together, these studies indicate a role for CaMKII and synapsin I in mechanisms associated with neurotransmitter release in drug dependence.

The current study focuses on investigating the changes in CaMKII and synapsin I function after nicotine withdrawal, and provides initial results, which suggest that the changes in function of these two proteins are relevant to nicotine withdrawal behaviors. Using CaMKII ^{32} P activity assays and western blot analysis, we measured nicotine-induced changes in CaMKII and synapsin I function in the VTA and NAc. The VTA and NAc are part of the mesocorticolimbic drug pathway, and several studies have implicated these two brain regions as having a role in mediating behaviors associated with nicotine dependence. Before proceeding with our withdrawal biochemical studies, we began with an acute nicotine assessment to determine how CaMKII and synapsin I are regulated by nicotinic receptors in the aforementioned brain regions. Although studies suggest an important role for nAChRs in the VTA in mediating nicotine withdrawal behaviors, the DA terminals that project from the VTA and the presynaptic nAChRs that mediate neurotransmitter release are located in the NAc. It is proposed that the decreased DA release following cessation of nicotine exposure is responsible for many of the mood disorders, craving, and anhedonia that persist in many smokers long after quitting (Benowitz, 2008). Because decreased DA release occurs in the NAc following precipitated and spontaneous withdrawal (Hildebrand et al., 1998; Rada et al., 2001), our chronic and withdrawal studies focus on CaMKII and synapsin I function in the NAc. To identify these changes in protein function, we used various nAChR antagonists to examine the acute nicotine-induced effects of nicotine in the VTA and NAc, followed by an assessment of nicotine's chronic effects on CaMKII and synapsin I function in the NAc. For the withdrawal evaluation in the NAc, we precipitated withdrawal using the β2-selective

antagonist, DHβE, and the α7 antagonist, MLA, and complemented our pharmacological approach using coinciding nAChR KO mice. Overall, the current assessment provides initial targets, which aid in the elucidation of post-receptor molecular mechanisms that may contribute to nicotine withdrawal behaviors.

B. Methods

Animals

Male B6 mice, β2 KO mice, and α7 KO mice and respective WT littermates were obtained and bred as described in Chapters 3 and 4.

Drugs

(-)-Nicotine hydrogen tartrate salt, mecamylamine hydrochloride, DHβE, and MLA were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Nimodipine was purchased from Research Biochemical International (Natick, MA). Nimodipine was dissolved in a vehicle solution made of 5% ethyl alcohol, 5% emulphor oil, and 90% saline. Drugs were administered at a volume of 10 ml/kg body weight. Doses are expressed as the free base of the drug.

Chronic nicotine administration protocol

Mice were implanted with Alzet osmotic mini pumps [model 2002 (14 days) or model 2004 (28 days) Durect Corporation, Cupertino, CA] filled with saline or (-)-nicotine (36 mg/kd/day) as described in Chapter 2.

Biochemical studies

Acute studies

Mice were treated with various antagonists [mecamylamine (2 mg/kg, s.c.), DHβE (2 mg/kg, s.c.), nimodipine (10 mg/kg, i.p.)] or saline prior to nicotine (2 mg/kg, s.c.) administration. This dose of nicotine was chosen because our studies found that there was a significant increase in CaMKII activity in the VTA and NAc after treatment with this

dose of nicotine (Fig. 29). The VTA and NAc were dissected 10 minutes after saline, mecamylamine, or DHβE injection, or 15 minutes after nicotine or nimodipine injection, and placed immediately in cold extraction buffer.

Chronic studies

Mice were chronically infused with nicotine for 14 days as described in the chronic administration protocol. The NAc was dissected on day 14 and placed immediately in cold extraction buffer.

Withdrawal studies

Chronic nicotine and saline infused mice were injected with DHβE (2 mg/kg, s.c.) or MLA (10mg/kg, s.c.) to precipitate withdrawal. nAChR KO mice were injected with mecamylamine (2 mg/kg, s.c.). The NAc was dissected 10 minutes after antagonist injection. Brain sections were placed in cold extraction buffer immediately after dissection.

CaMKII ^{32} P activity assay

CaMKII activity was measured using an assay kit (Upstate biotechnology, Lake Placid, NY). Following experimental treatment, brain tissues were homogenized in Ca^{2+}-free Tris buffer that contains 1 mM PMSF. Homogenates were normalized for protein concentration. Samples were centrifuged in order to separate the membrane and the cytosol containing-kinase. The activity of CaMKII in the membrane portion was measured.

Standard phosphorylation reaction solutions contained 70 µg protein, 10 mM MgCl2, 1 µCi of [32P]ATP, 10 mM Pipearzine-*N*,*N*'-bis(2-ethanesulfonic acid) (PIPES) (pH 7.4), 5 µM CaCl2 and 5 µg calmodulin. Standard reactions were performed in a shaking water bath at 30°C. Ca^{2+}-dependent calmodulin-protein kinase II activity was determined using the following calculations: [(count-specific binding) x (correcting factor)]/[(specific radioactivity)x time (10 min)]. Activity was expressed as the number of pmol ^{32}P incorporated into calmodulin protein kinase II substrate peptide/min/mg of protein in the presence (dependent reaction) or absence (independent reaction).

Western blot assay

VTA and NAc sections were homogenized in extraction buffer containing 50 mM Tris, 1 % SDS, 1 mM PMSF, 1 mM EDTA, 5 mM EGTA, 1 mM Na+ orthovanadate, 20 µg/ml leupeptin, 10 µg/ml aprotinin, and 1 µM okadaic acid . Protein concentrations were determined using the Bradford assay and 20 µg of protein were incubated with sample buffer and heated for 5 minutes at 95°C. Samples were then separated by SDS-polyacrylamide gel electrophoresis on a 10% TRIS-HCL gel and subjected to immunoblotting. α-CaMKII (1:1000; Sigma, St. Louis, MO), α-pCaMKII (1:10000; Fisher Scientific, USA), Synapsin I (1:2000; Chemicon International, Inc, USA), or pSynapsin I Ser 603 (an antibody specific for the site phosphorylated by CaMKII) (1:2000; Sigma, St. Louis, MO) primary antibodies and α-tubulin antibody (1:5000; Upstate, Temecula, CA) were incubated overnight at 4°C. Secondary antibodies (1:5000; LiCor Biosciences, Inc., Lincoln, NE) were incubated for 1 hour at room temperature. Bound antibody was detected using the LiCor Odyssey Infrared Imaging System (LiCor Biosciences, Inc., Lincoln, NE).

α-CaMKII bands were detected at 50 kDa, α-pCaMK II bands were detected at 52 kDa, Synapsin I bands were detected at 80 kDa, pSynapsin I Ser 603 bands were detected at 78 kDa, and α-tubulin bands were detected at 55 kDa.

Staistical analysis

For all data, statistical analyses were performed using StatView ® (SAS, Cary, NC, USA). Data were analyzed with one-way ANOVAs with treatment as the between subject factor or two-way ANOVAs with treatment and genotype as between subject factors. Significant results were further analyzed using the Neuman-Keuls post-hoc test. *p* values of less than 0.05 were considered significant.

C. Results

Effect of acute nicotine administration on CaMKII activity in the VTA and NAc

Before starting our withdrawal assessment, we first wanted to determine how CaMKII is regulated after acute nicotine, as acute drug responses are an important part of the initial cascade that occurs in response to drug exposure. CaMKII activity was measured in the VTA and NAc after acute systemic nicotine administration (2 mg/kg, s.c.). Results show that acute nicotine induces increases in CaMKII activity in the VTA (Fig. 29A) and NAc (Fig. 29B).

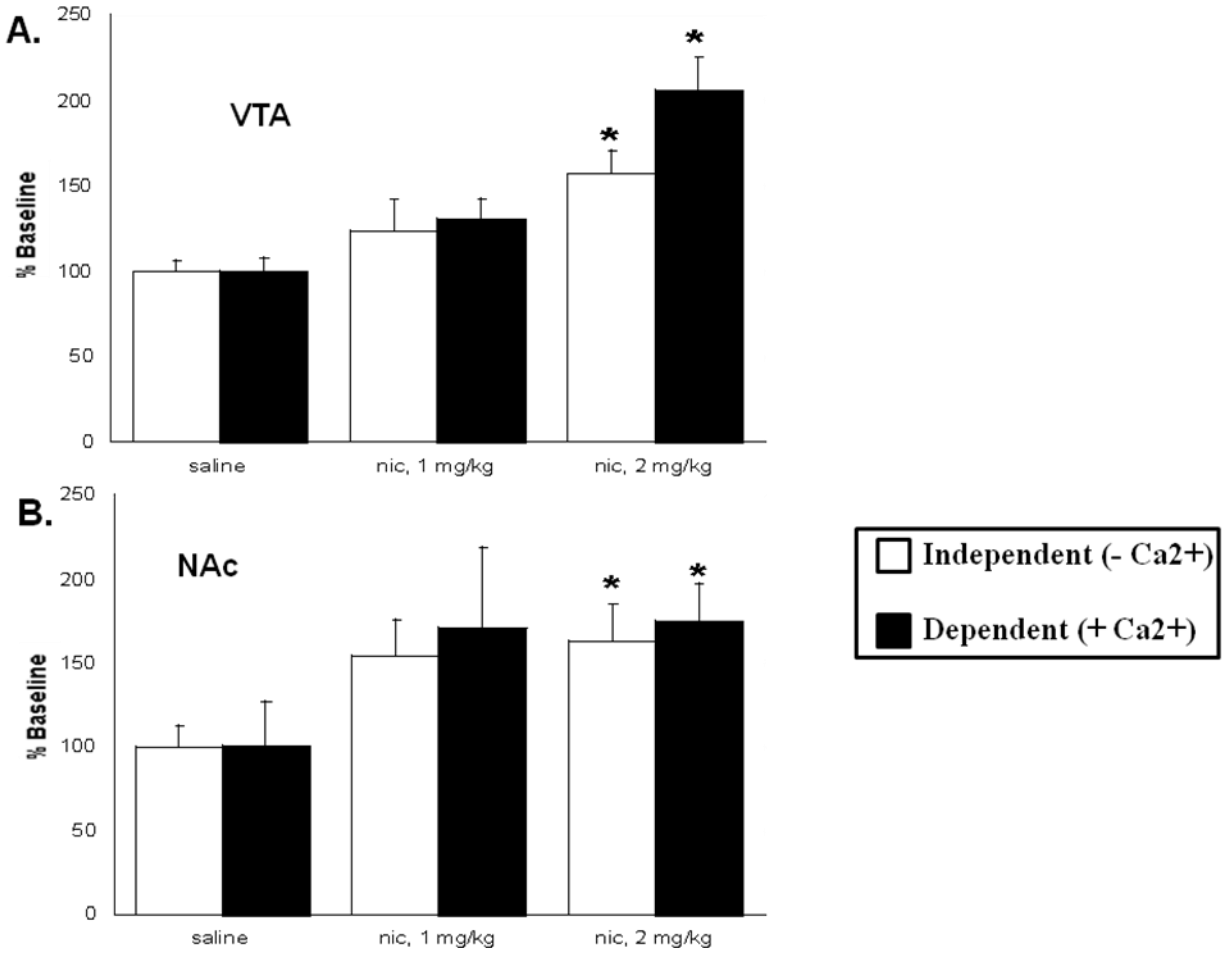


Figure 29. Acute nicotine induces increases in CaMKII activity in the VTA and NAc. Mice were treated with an acute systemic injection of nicotine (2 mg/kg, s.c.), and brain sections were dissected and prepared for analysis using a CaMKII ^{32} P activity assay. Activity was expressed as the number of pmol ^{32}P incorporated into calmodulin protein kinase II substrate peptide/min/mg of protein in the presence (dependent) or absence (independent) of calcium. Each point represents the mean ± S.E.M. of 5 mice per group. * denotes p < 0.05 vs. saline group

Next, we wanted to determine if the nicotine-induced increase in CaMKII activity was mediated through nAChRs, and examine the involvement of L-type VGCCs, which may suggest a role for indirect sources of nicotine-induced Ca^{2+} influx. Mice were treated with the non-selective nAChR antagonist, mecamylamine (2 mg/kg, s.c.), or the L-type VGCC blocker, nimodipine (10 mg/kg, i.p.), 10 or 15 minutes prior to nicotine administration. Mecamylamine and nimodipine significantly blocked the acute nicotine-induced increase in CaMKII activity in the VTA (Fig. 30), suggesting that the nicotine-induced increase in CaMKII activity is mediated directly through nAChRs and involves indirect sources of nicotine-induced Ca^{2+} influx through L-type VGCC channels. The antagonist doses used did not produce a significant effect alone, as there was no significant difference in activity between naïve antagonist treated mice and saline treated mice (Fig. 30).

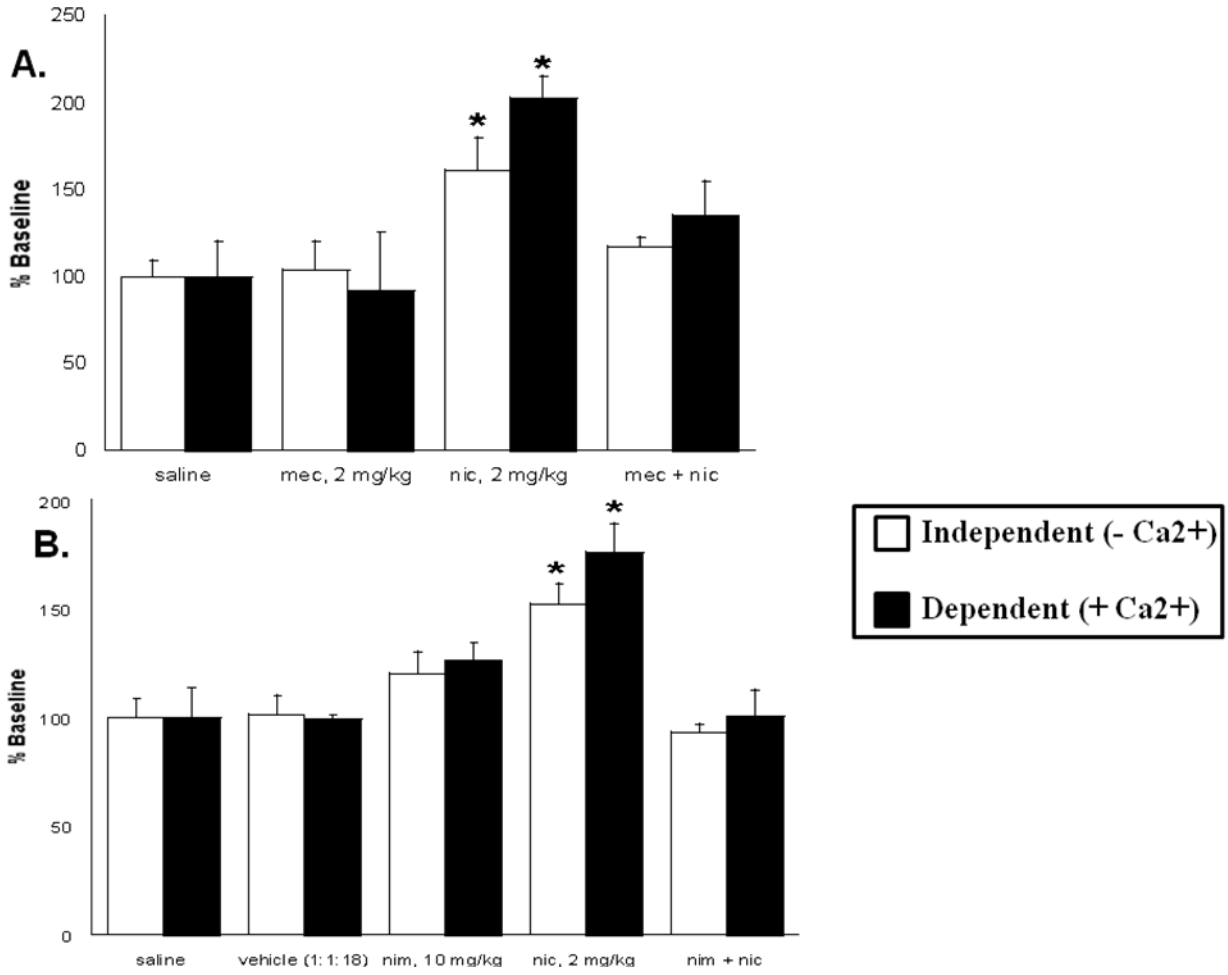


Figure 30. Acute nicotine induces an increase in CaMKII activity in the VTA that is mediated directly through nAChRs and indirectly through L-type VGCCs. Mice were treated with mecamylamine (2 mg/kg, s.c.) or nimodipine (10 mg/kg, i.p.) 10 or 15 minutes prior to an acute systemic injection of nicotine (2 mg/kg, s.c.) Brain sections were dissected and prepared for analysis using a CaMKII ^{32} P activity assay. Activity was calculated as the number of pmol ^{32}P incorporated into calmodulin protein kinase II substrate peptide/min/mg of protein in the presence (dependent) or absence (independent) of Ca^{2+}, and expressed as % deviation from baseline response. Each point represents the mean ± S.E.M. of 5 mice per group. * denotes $p < 0.05$ vs. saline, mec, and mec + nic groups

Synapsin I activity in the VTA and NAc after acute nicotine administration

Studies in the lab revealed that the acute nicotine induced increase in CaMKII activity in the VTA and NAc was significantly blocked by DHβE, but not MLA (Walters and Damaj, 2008, submitted). Further, the acute nicotine-induced increase in CaMKII activity was present in α7, but not β2 KO mice (Walters and Damaj, 2008, submitted). These results suggest that the acute nicotine-induced increases in CaMKII activity are mediated by β2-containing, and not α7, nAChRs. Western blot analysis also revealed that the total CaMKII protein level was unchanged after acute nicotine treatment (Walters and Damaj, 2008, submitted). Synapsin I is a substrate of CaMKII that is phosphorylated by the kinase at Ser-566 and Ser-603; thus, we wanted to see if synapsin I plays a role in the acute pharmacological effects of nicotine. Western blot analysis revealed a significant increase in pSynapsin I Ser 603 (p603) in the VTA (Fig. 31) and NAc (Fig. 32) after acute nicotine administration (2 mg/kg, s.c.). The acute nicotine-induced increases in p603 in the VTA (Fig. 31) and NAc (Fig. 32) were significantly blocked by mecamylamine, suggesting a nAChR-mediated effect. To test the specificity of this effect, we treated mice with the β2-selective antagonist, DHβE (2 mg/kg, s.c.) 10 minutes before nicotine administration. Similar to what was observed with pCaMKII activity, results show that the acute nicotine-induced increase in p603 in the VTA (Fig. 33) and NAc (Fig. 34) were significantly blocked by DHβE at doses that are not biochemically active alone. There was no change in the total synapsin I protein level in either the VTA or NAc with any drug treatment (Figs. 31-34).

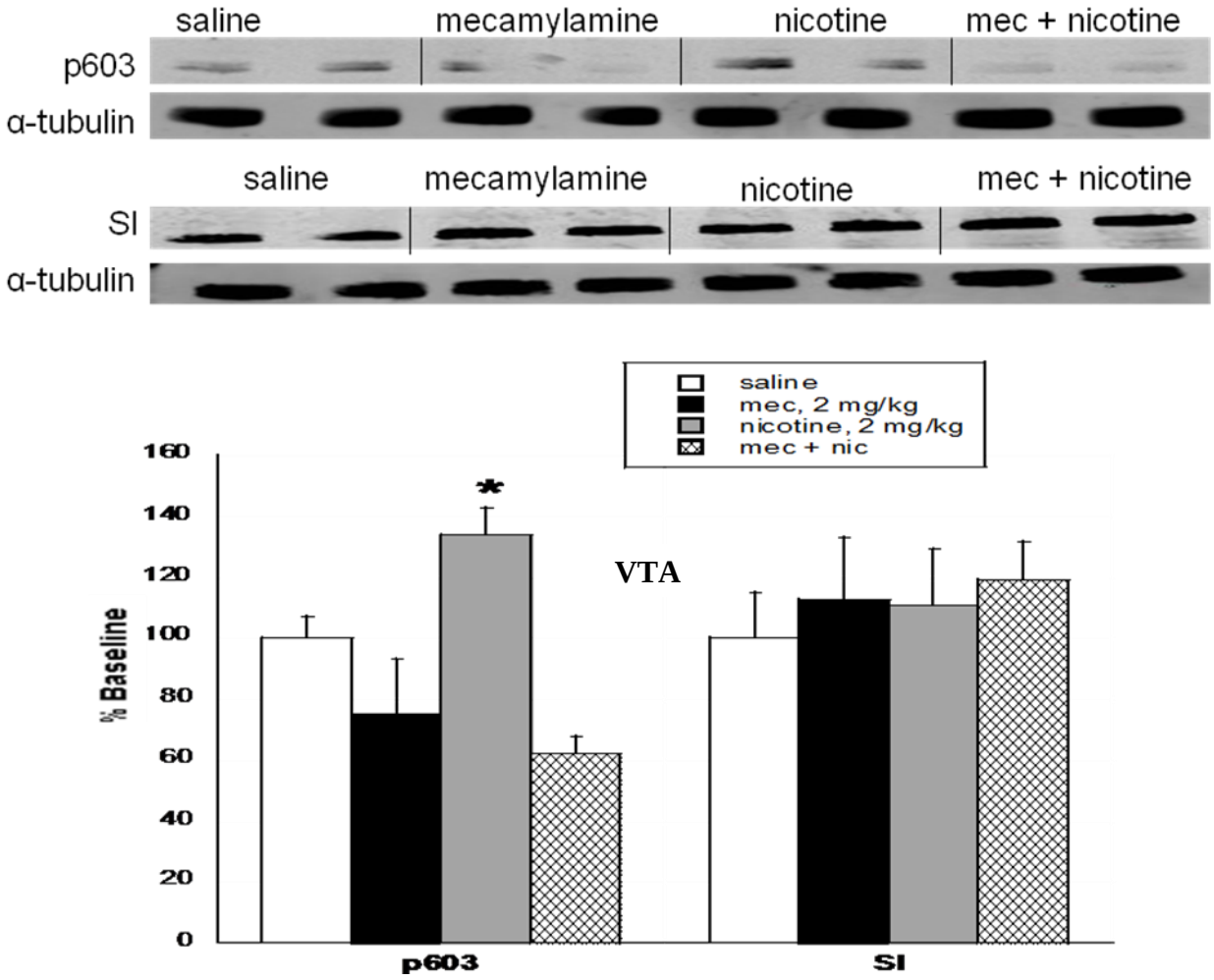


Figure 31. Acute nicotine-induced increases in pSynapsin I Ser 603 (p603) in the VTA are blocked by mecamylamine. The dose of mecamylamine (mec, 2 mg/kg, s.c.) used was not active by itself. Results were calculated as the ratio of p603 or synapsin (SI) and α-tubulin and expressed as the % deviation from the baseline response. Each point represents the mean ± S.E.M. of 4 mice per group. * denotes $p < 0.05$ vs. saline, mec, and mec + nic groups

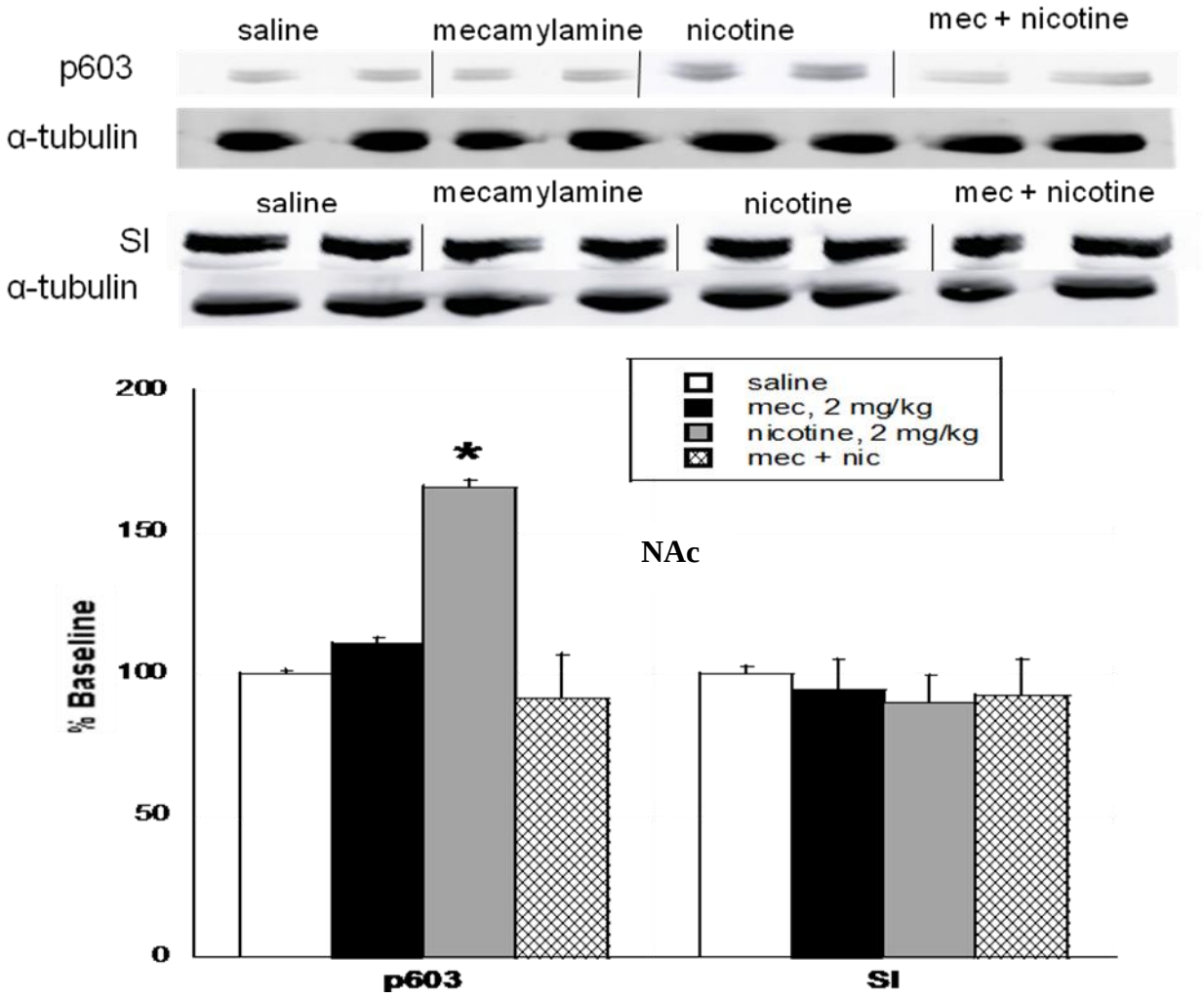


Figure 32. Acute nicotine-induced increases in pSynapsin I Ser 603 (p603) in the NAc are blocked by mecamylamine. The dose of mecamylamine (mec, 2 mg/kg, s.c.) used was not active by itself. Results were calculated as the ratio of p603 or synapsin (SI) and α-tubulin and expressed as the % deviation from the baseline response. Each point represents the mean ± S.E.M. of 4 mice per group. * denotes $p < 0.05$ vs. saline, mec, and mec + nic groups

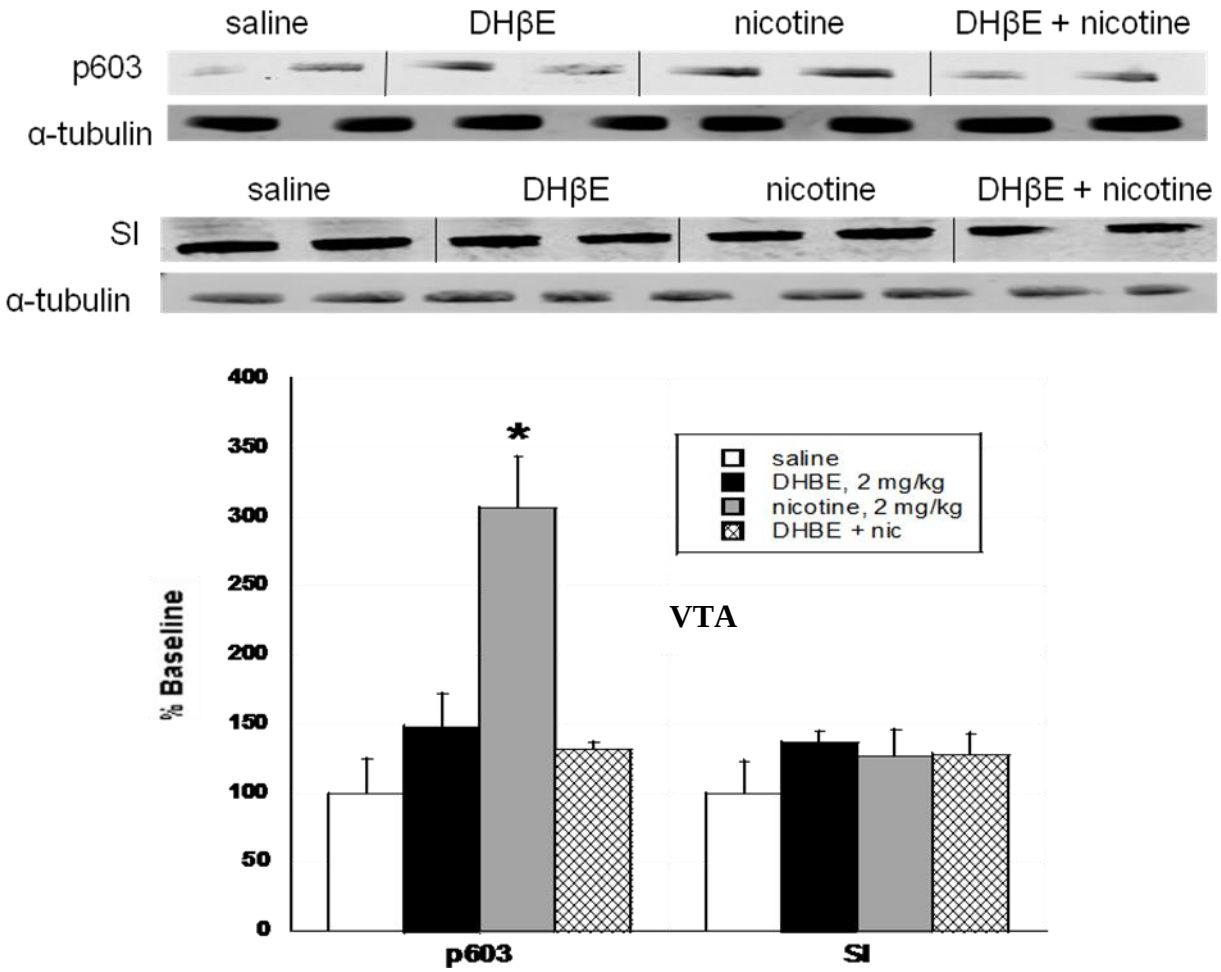


Figure 33. Acute nicotine-induced increases in pSynapsin I Ser 603 (p603) in the VTA are blocked by the β2-selective antagonist, DHβE. The dose of DHβE (2 mg/kg, s.c.) used was not active by itself. Results were calculated as the ratio of p603 or synapsin (SI) and α-tubulin and expressed as the % deviation from the baseline response. Each point represents the mean ± S.E.M. of 4 mice per group. * denotes $p < 0.05$ vs. saline, DHβE, and DHβE + nic groups

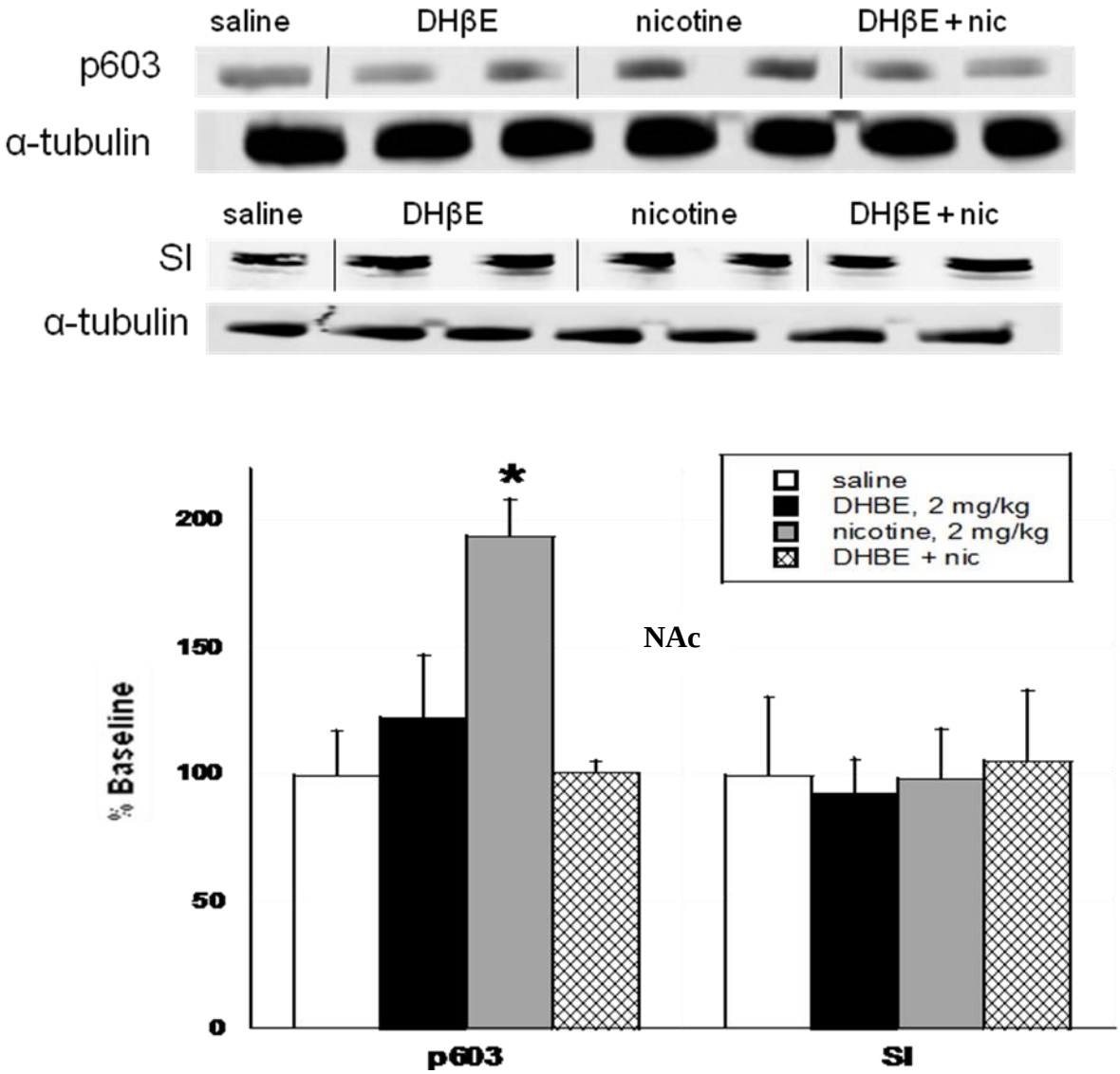


Figure 34. Acute nicotine-induced increases in pSynapsin I Ser 603 (p603) in the NAc are blocked by the β2-selective antagonist, DHβE. The dose of DHβE (2 mg/kg, s.c.) used was not active by itself. Results were calculated as the ratio of p603 or synapsin (SI) and α-tubulin and expressed as the % deviation from the baseline response. Each point represents the mean ± S.E.M. of 4 mice per group. * denotes $p < 0.05$ vs. saline, DHβE, and DHβE + nic groups

Synapsin I activity in the NAc after chronic nicotine exposure

In order to understand the mechanisms involved in nicotine withdrawal, we first needed to examine changes in activity after chronic nicotine exposure. Mice were chronically infused with nicotine for 14 days. On day 14, the NAc was dissected and prepared for western blot analysis. Results show that chronic nicotine induces a significant increase in p603 expression compared to chronically infused saline controls (Fig. 35), suggesting that tolerance does not develop to the acute nicotine-induced increase in p603.

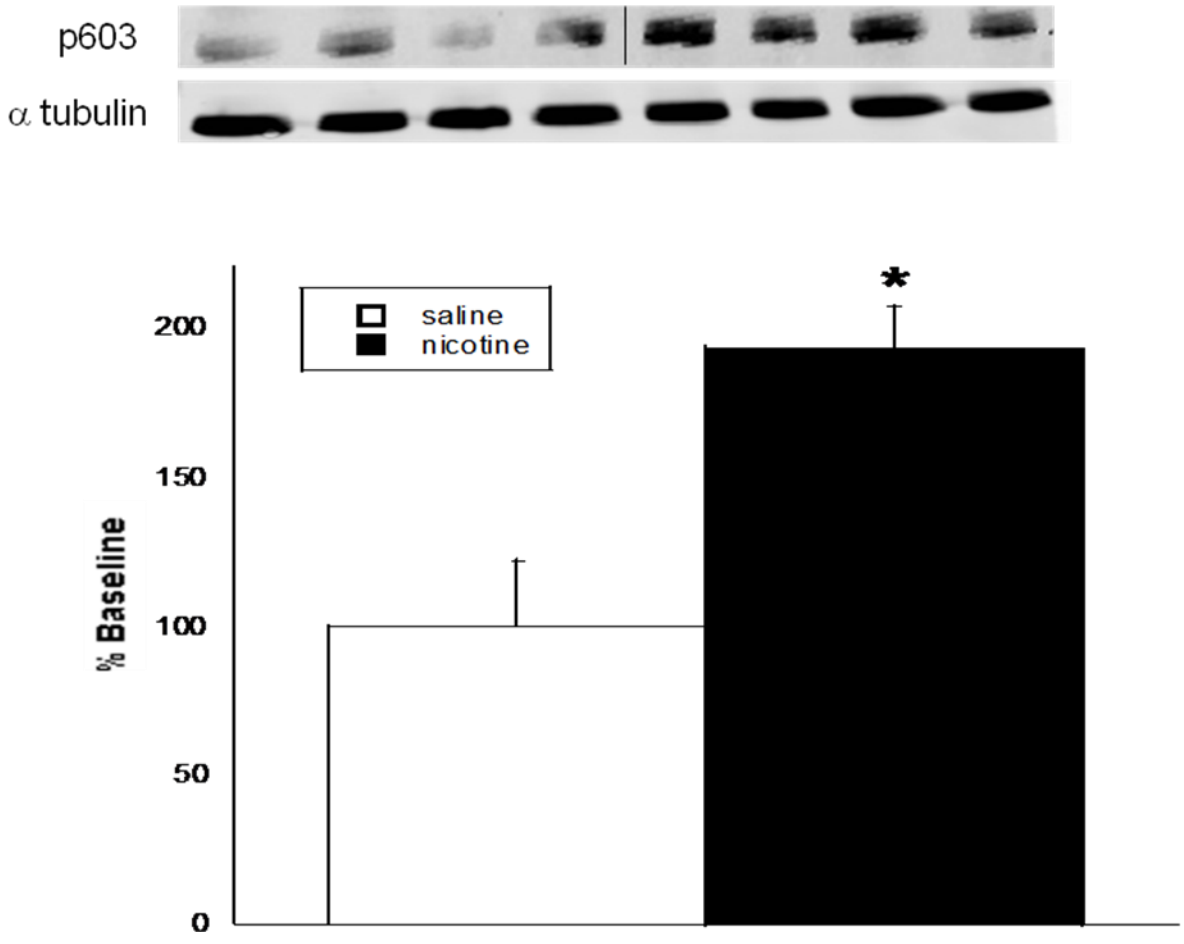


Figure 35. Chronic nicotine induces an increase in pSynapsin I Ser 603 (p603) activity in the NAc. After 14 days of chronic nicotine exposure, there was a significant increase in p603 expression in the NAc compared to chronic saline controls. Each point represents the mean ± S.E.M. of 4 mice per group. * denotes $p < 0.05$ vs. saline

CaMKII and Synapsin I activity in the NAc after nicotine withdrawal

Stimulation of nAChRs in the VTA leads to alterations in neurotransmitter release in the NAc. Our data show that acute nicotine increases activity of important markers of neurotransmitter release (CaMKII and synapsin I). Further, after nicotine withdrawal, decreased DA release in the NAc was reported (Hildebrand et al., 1998; Rada et al., 2001), and it has been proposed that this deficient DA release in the NAc contributes to the mood disorders and overall negative affective state that accompanies nicotine withdrawal (Benowitz, 2008); therefore, we focused on changes in CaMKII and synapsin I function in the NAc after nicotine withdrawal. Based on the decreased DA release observed in the NAc after nicotine withdrawal, we hypothesized that there would be a decrease in CaMKII and synapsin I activity in the NAc after nicotine withdrawal. To assess changes in protein function, mice chronically infused with nicotine or saline for 14 days were treated with the β2-selective antagonist, DHβE (2 mg/kg, s.c.), or the α7 antagonist, MLA (10 mg/kg, s.c.), on the morning of day 15, and brain sections were dissected 10 minutes after injection. A spontaneous withdrawal assessment was also conducted to complement our precipitated biochemical results. Results show that DHβE (2 mg/kg, s.c.) at a dose that precipitates significant aversion in mice (Chapter 2) induced a significant decrease in pCaMKII and CaMKII total protein levels in the NAc (Fig. 36). Additionally, p603 and synapsin I total protein levels in the NAc of chronic nicotine infused mice were decreased after treatment with DHβE, coinciding with the decreased CaMKII function (Fig. 37). Conversely, MLA did not precipitate a change in pCaMKII, CaMKII (Fig. 38), p603 or synapsin I (Fig. 39)

function in the NAc, suggesting that α7 nAChRs are not involved in nicotine-withdrawal induced changes in this brain region.

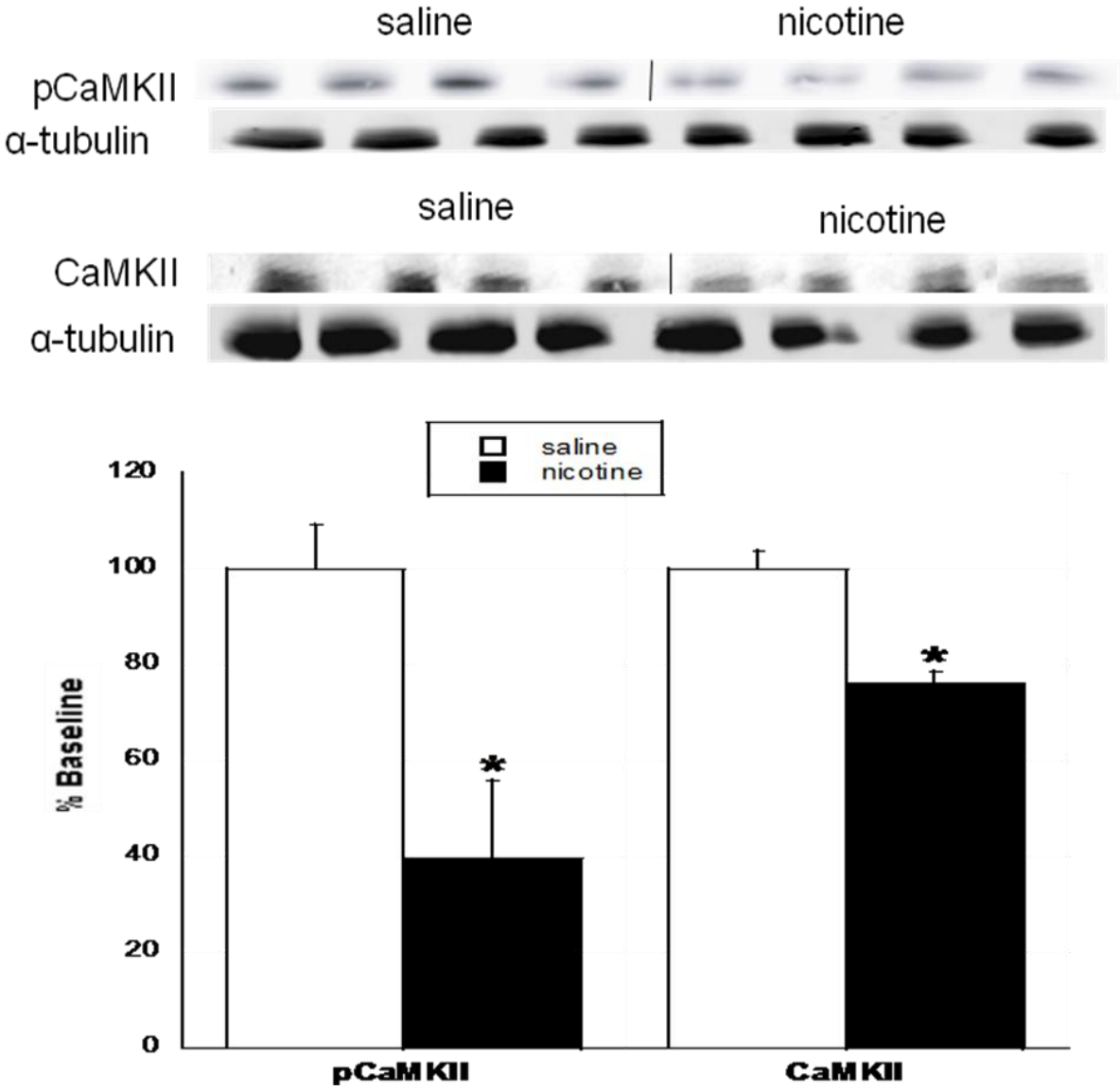


Figure 36. DHβE significantly reduces pCaMKII and CaMKII activity and level in the NAc after chronic nicotine administration. Mice chronically infused with nicotine for 14 days were injected with DHβE (2 mg/kg, s.c.) on the morning of day 15 to precipitate withdrawal. Each point represents the mean ± S.E.M. of 5 mice per group. * denotes $p < 0.05$ vs. saline group

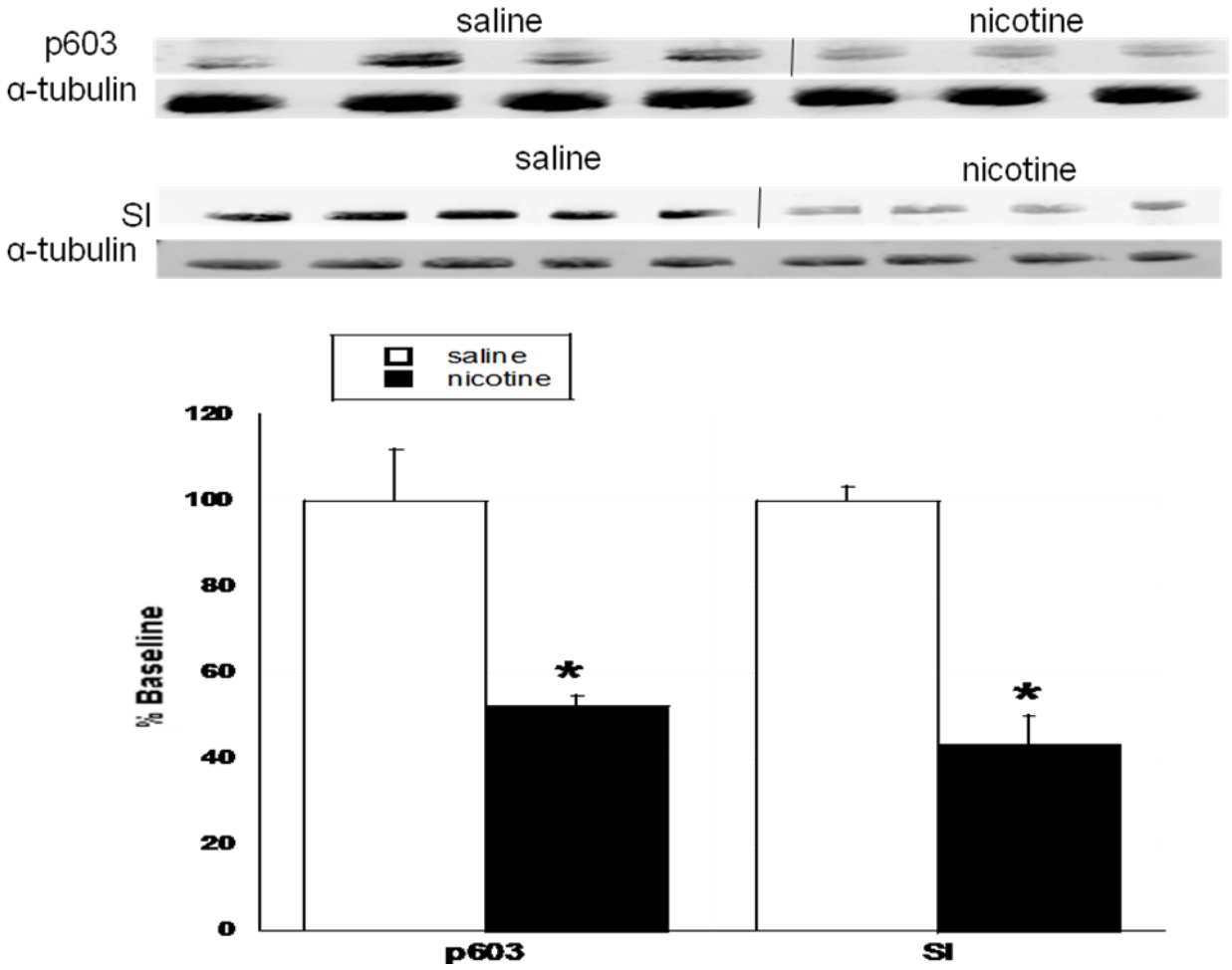


Figure 37. DHβE significantly reduces pSynapsin I Ser 603 (p603) and Synapsin I (SI) activity and level in the NAc after chronic nicotine administration. Mice chronically infused with nicotine for 14 days were injected with DHβE (2 mg/kg, s.c.) on the morning of day 15 to precipitate withdrawal. Each point represents the mean ± S.E.M. of 5 mice per group. *denotes $p < 0.05$ vs. saline group

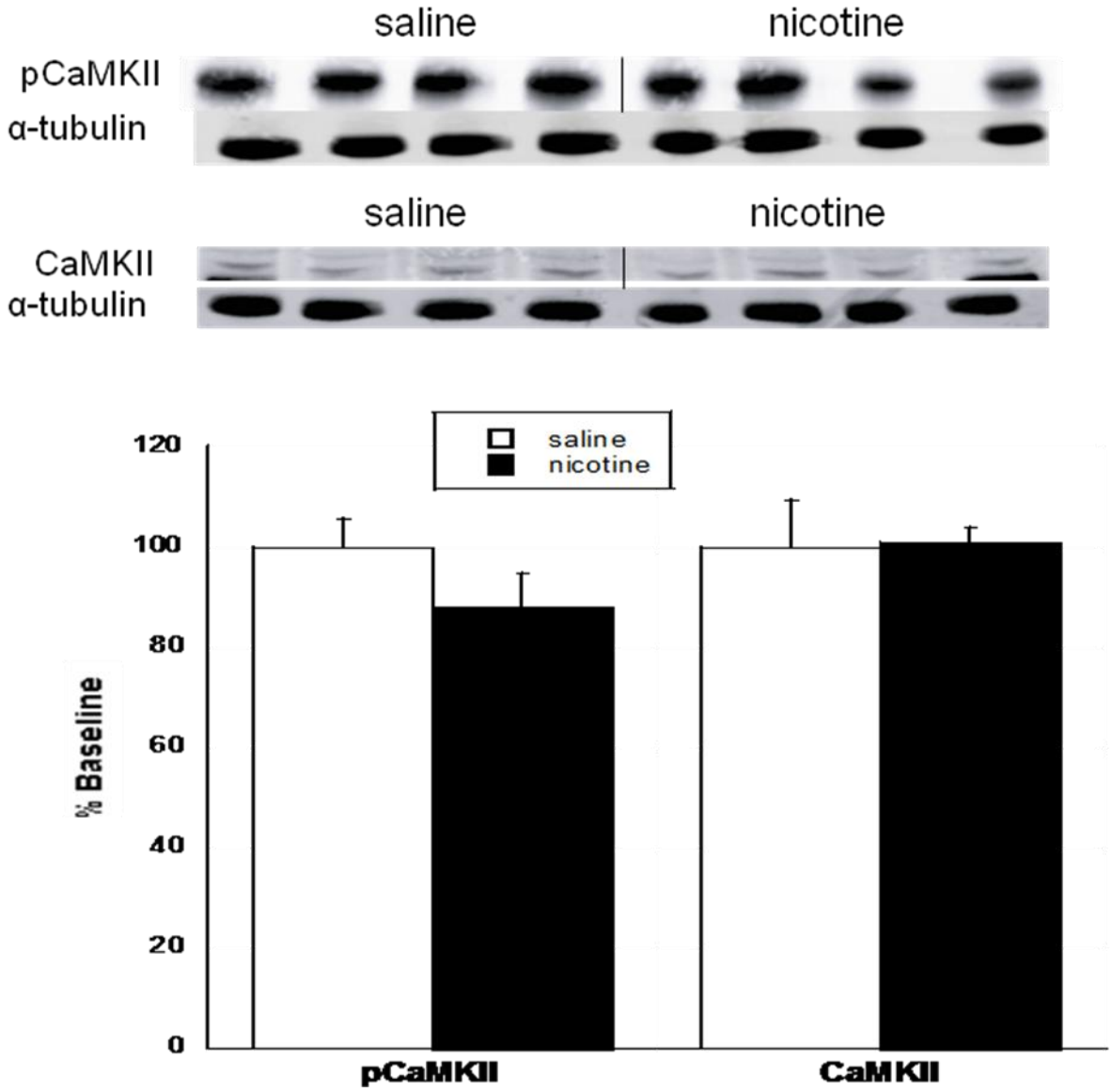


Figure 38. MLA does not precipitate a change in pCaMKII or CaMKII activity or level in the NAc. Mice chronically infused with nicotine for 14 days were injected with MLA (10 mg/kg, s.c.) on the morning of day 15 to precipitate withdrawal. There was no significant difference between saline and nicotine infused mice after MLA treatment. Each point represents the mean ± S.E.M. of 5 mice per group.

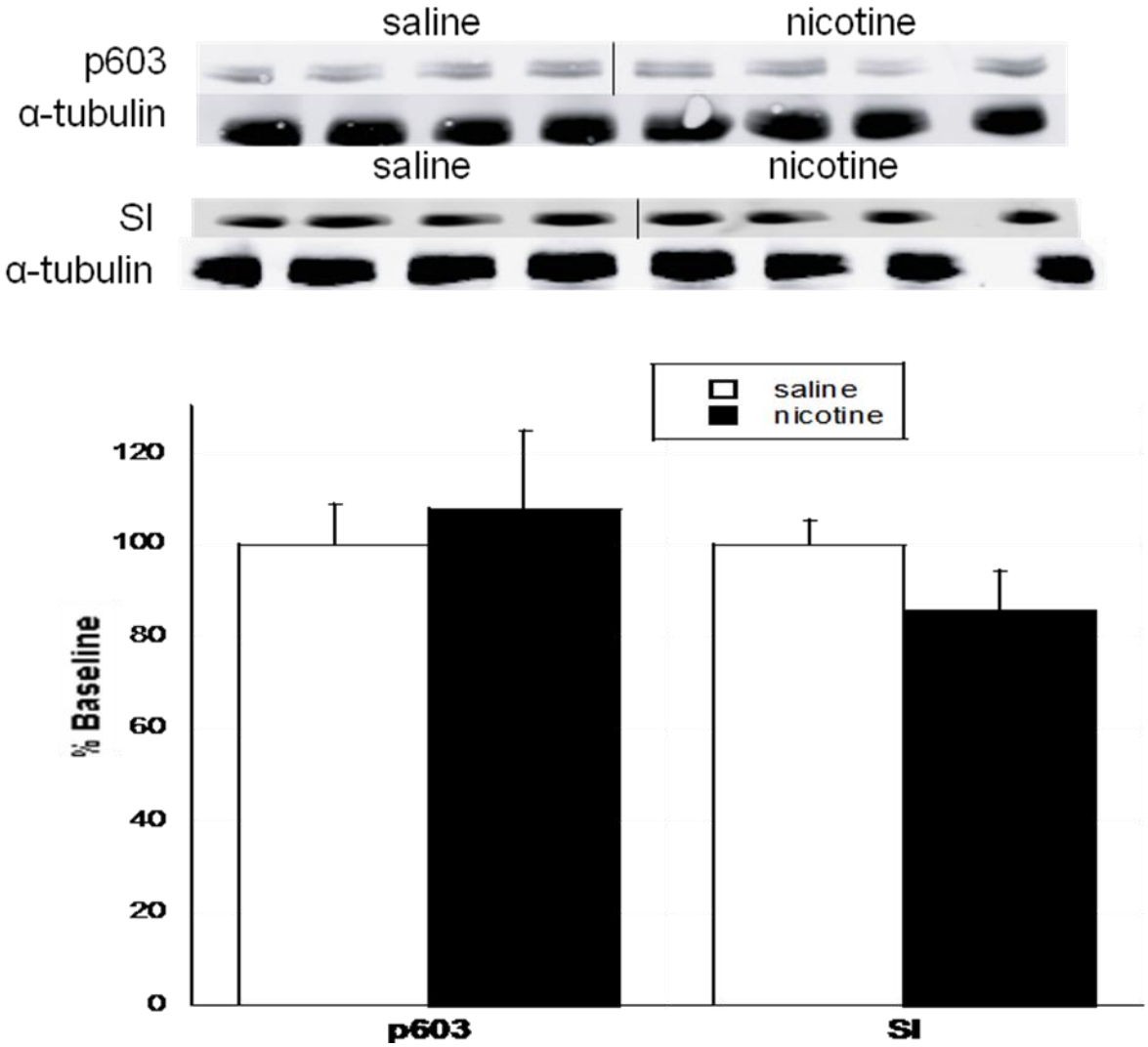


Figure 39. MLA does not precipitate a change in pSynapsin I Ser 603 (p603) or synapsin I (SI) activity or level in the NAc. Mice chronically infused with nicotine for 14 days were injected with MLA (10 mg/kg, s.c.) on the morning of day 15 to precipitate withdrawal. There was no significant difference between saline and nicotine infused mice after MLA treatment. Each point represents the mean ± S.E.M. of 5 mice per group.

To complement our pharmacological approach using DHβE and MLA, we examined protein function in β2 and α7 KO mice. β2 and α7 KO and WT mice were chronically infused with saline or nicotine for 14 days. On day 15, the mice were treated with mecamylamine (2 mg/kg, s.c.), and pCaMKII, CaMKII, p603, and synapsin I were measured in the NAc. There was a significant decrease in pCaMKII activity in chronic nicotine infused WT mice after mecamylamine treatment compared to saline counterparts; however, the decrease in pCaMKII activity was absent in β2 KO mice (Fig. 40). Additionally, the decrease in p603 activity in WT mice that was also absent in β2 KO mice (Fig. 41). Interestingly, there was no change in total CaMKII (Fig. 40) or synapsin I protein level (Fig. 41) in WT mice after mecamylamine treatment in any group tested.

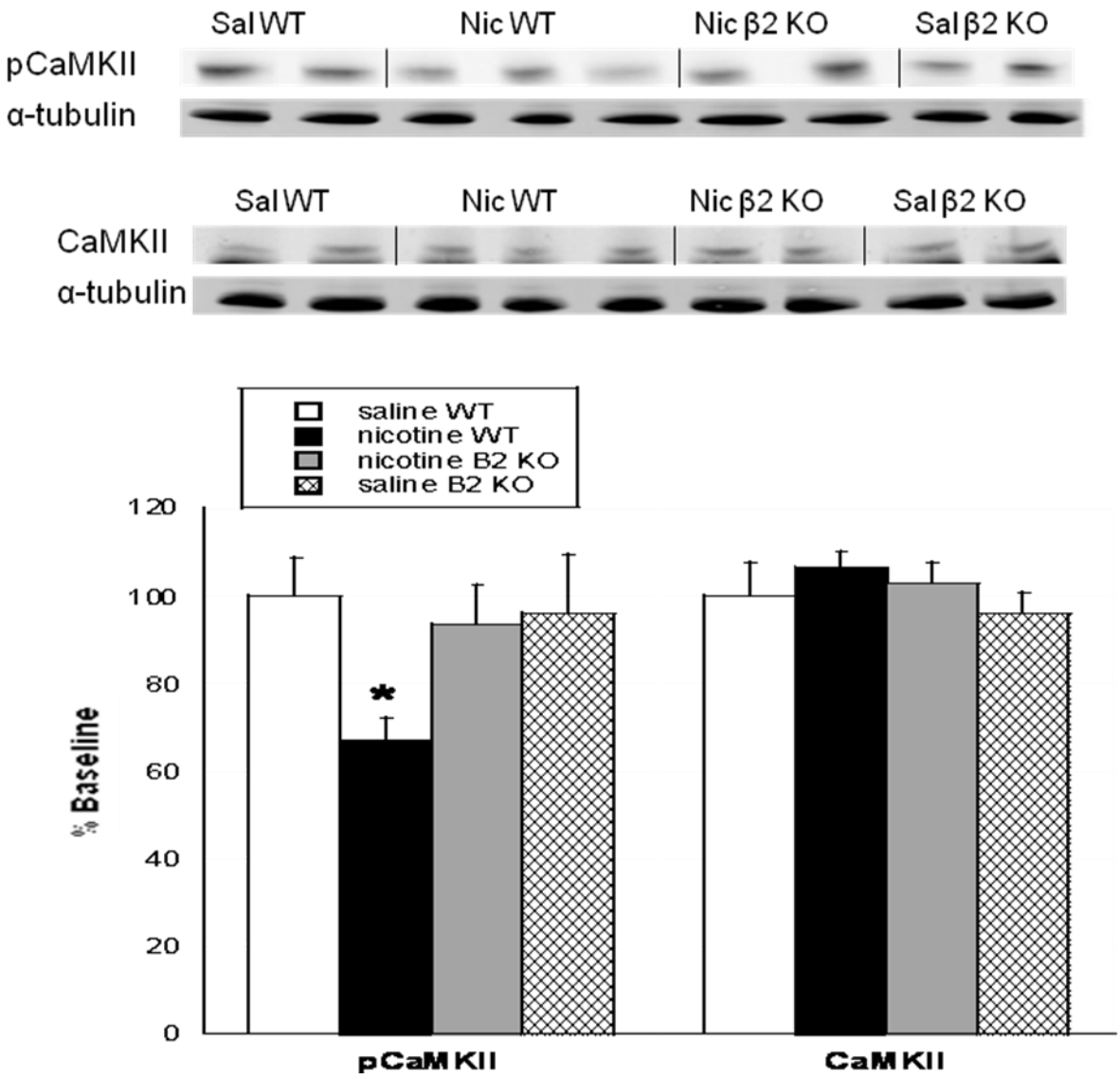


Figure 40. Assessment of pCaMKII and CaMKII activity and level in β2 KO mice. β2 KO and WT mice chronically infused with nicotine for 14 days were injected with mecamylamine (mec, 2 mg/kg s.c.) on the morning of day 15 to precipitate withdrawal. There was a significant decrease in pCaMKII activity in WT mice that was not present in β2 KO mice. Interestingly, there was no change in total CaMKII protein level in any group tested after mecamylamine treatment. Each point represents the mean ± S.E.M. of 4-6 mice per group. * denotes $p < 0.05$ vs. saline and β2 KO groups

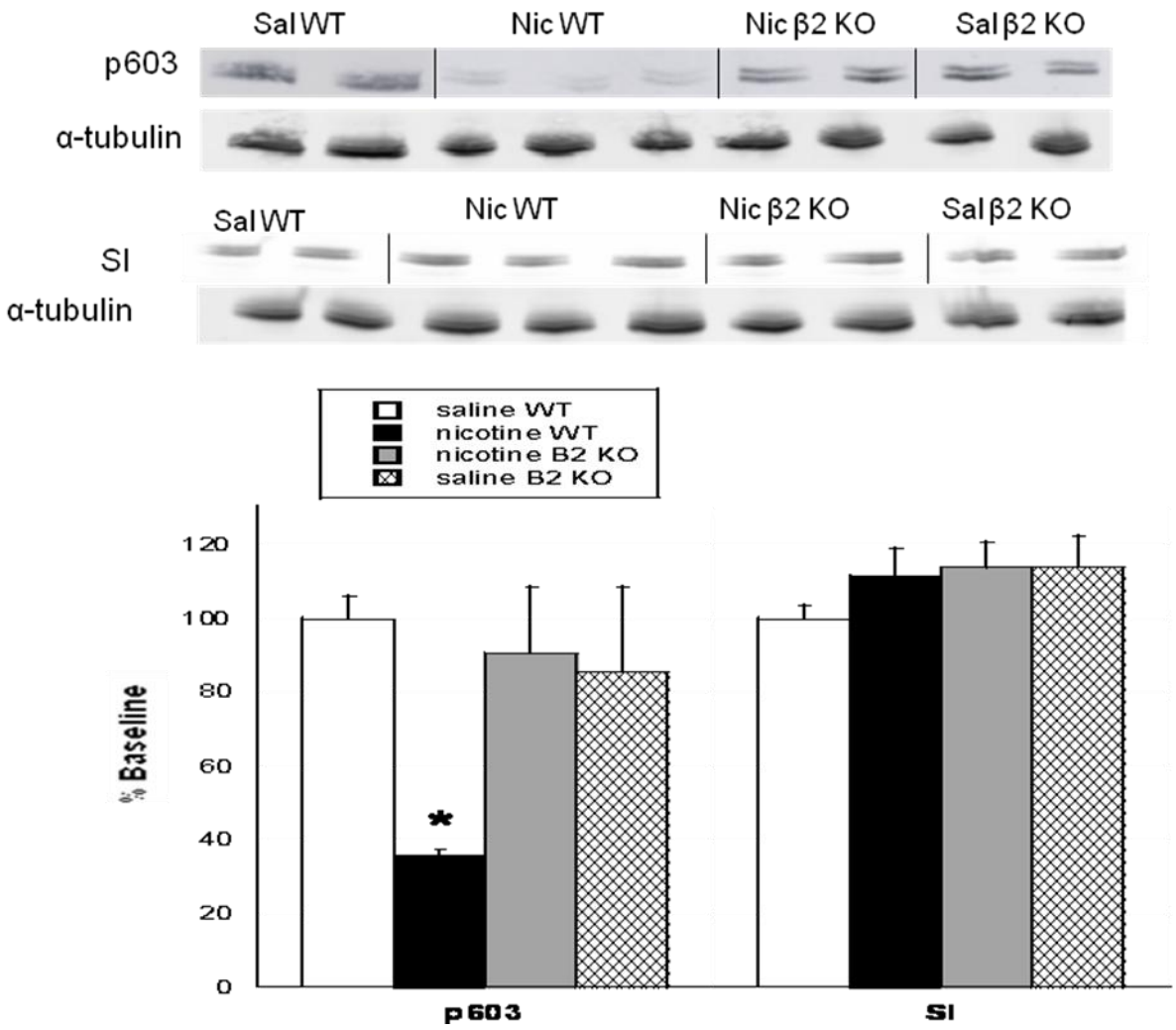


Figure 41. Assessment of pSynapsin I Ser 603 (p603) and synapsin I (SI) activity and level in β2 KO mice. β2 KO and WT mice chronically infused with nicotine for 14 days were injected with mecamylamine (mec, 2 mg/kg s.c.) on the morning of day 15 to precipitate withdrawal. There was a significant decrease in p603 activity in WT mice that was not present in β2 KO mice. Interestingly, there was no change in total synapsin I protein level in any group tested after mecamylamine treatment. Each point represents the mean ± S.E.M. of 4-6 mice per group. * denotes $p < 0.05$ vs. saline and β2 KO groups

MLA, an α7 antagonist, did not precipitate a decrease in CaMKII or synapsin I function, suggesting that α7 nAChRs are not involved in the nicotine withdrawal-induced change in CaMKII or synapsin I function. To complement the results of this pharmacological study, we measured protein levels in nicotine-dependent α7 nAChR WT and KO mice after mecamylamine treatment. The assessment in α7 KO mice revealed a significant decrease in pCaMKII (Fig. 42) and p603 activity (Fig. 43) in both nicotine-infused α7 WT and KO mice after mecamylamine treatment. There was no change in the total protein level for either CaMKII or synapsin I in any group tested (Figs. 42 and 43).

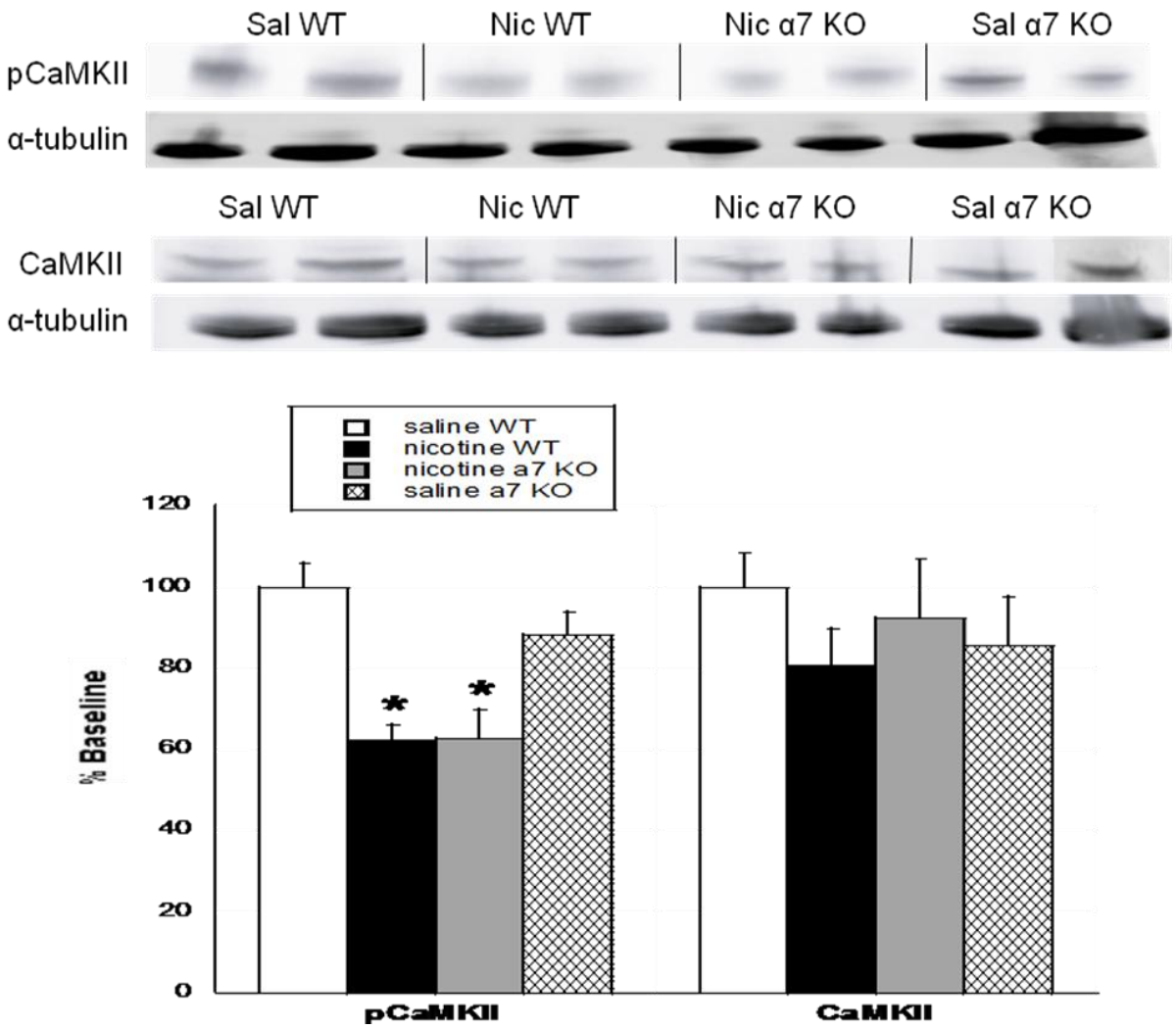


Figure 42. Assessment of pCaMKII and CaMKII function in α7 KO mice. α7 KO and WT mice chronically infused with nicotine for 14 days were injected with mecamylamine (mec, 2 mg/kg s.c.) on the morning of day 15 to precipitate withdrawal. There was a significant decrease in pCaKII activity in α7 KO and WT mice. Interestingly, there was no change in total CaMKII protein level in any group tested after mecamylamine treatment. Each point represents the mean ± S.E.M. of 4-6 mice per group. * denotes $p < 0.05$ vs. saline groups

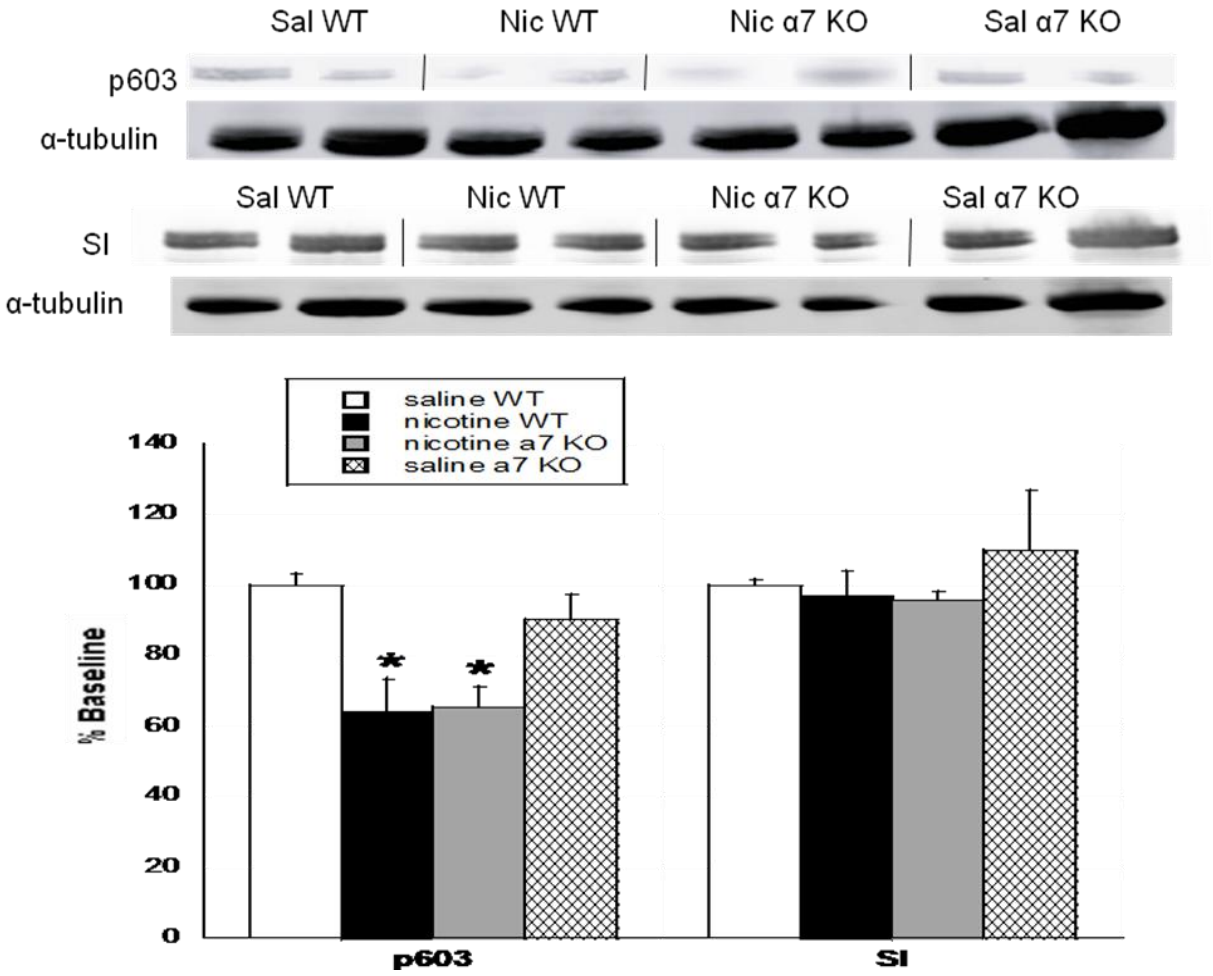


Figure 43. Assessment of pSynapsin I Ser 603 (p603) and synapsin I function in α7 KO mice. α7 KO and WT mice chronically infused with nicotine for 14 days were injected with mecamylamine (mec, 2 mg/kg s.c.) on the morning of day 15 to precipitate withdrawal. There was a significant decrease in p603 activity in α7 KO and WT mice with no change in total synapsin I protein level in any group tested after mecamylamine treatment. Each point represents the mean ± S.E.M. of 4-6 mice per group. * denotes $p < 0.05$ vs. saline groups

D. Discussion

The goal of the biochemical studies in this chapter was to examine the relationship between *in vivo* behavioral modifications and molecular mechanisms that mediate nicotine withdrawal behaviors. The current studies suggest that β2-containing nAChRs, but not α7 nAChRs, are crucial in mediating the decreases in CaMKII and synapsin I activity in the NAc after nicotine withdrawal. DHβE blocked the increase in synapsin I activity in the VTA and NAc. Additionally, DHβE, but not MLA, precipitated a significant decrease in CaMKII and synapsin I activity and protein level. These results were complemented using β2 and α7 nAChR KO, where it was shown that mecamylamine precipitated a significant decrease in CaMKII and synapsin I function in WT and α7 KO mice, but the decrease was not present in β2 KO mice after 14 days of chronic nicotine infusion.

We began our studies by determining the acute effect of nicotine on CaMKII and synpasin I function, as this represents the initial cascade of events that occurs after acute nicotine exposure. Our studies revealed that acute systemic nicotine induced increases in CaMKII activity in the VTA and NAc. The increased activity was significantly blocked by the non-selective nAChR antagonist, mecamylamine, as well as by the L-type VGCC blocker nimodipine in the VTA. While these results indicate that the acute nicotine-induced increase in CaMKII activity is mediated directly through nAChR, it also suggests an important role for L-type VGCC, which may suggest the importance of indirect sources of Ca^{2+} influx in nicotine induced CaMKII activation. It must be noted that *in vitro* studies show that L-type VGCC blockers can block nAChR signaling at doses typically used to

block L-type VGCCs (Wheeler et al., 2006); thus, these results should be interpreted with caution, as they may reflect actions at targets other than L-type VGCC.

We also examined the CaMKII substrate, synapsin I, a vesicle associated protein essential for neurotransmitter release, after acute nicotine. As seen with CaMKII, there was a significant increase in synapsin I activity, but not total protein level after acute nicotine treatment. This nicotine-induced increase in activity was significantly blocked by mecamylamine, and by DHβE in the VTA and NAc at doses used to precipitate affective nicotine withdrawal behaviors (Chapter 2 and 3). Previous studies from our lab also show that acute nicotine-induced increases in CaMKII in the VTA and NAc are mediated by β2*, but not α7* nAChRs (Walters et al., 2008, submitted). Taken together, these results imply that acute nicotine-induced increases in CaMKII and synapsin I activity are mediated by β2-containing nAChRs in the VTA and NAc.

Our chronic nicotine assessment revealed a significant increase in p603 expression after 14 days of chronic nicotine exposure. These results suggest that tolerance does not develop to the acute nicotine-induced increases in p603. In addition to the acute and chronic nicotine studies, we conducted a withdrawal assessment, which suggests an important role for β2*, but not α7* nAChRs in nicotine withdrawal. Although studies suggest an important role for nAChRs in the VTA in mediating nicotine withdrawal behaviors, the DA terminals that project from the VTA and the presynaptic nAChRs that mediate neurotransmitter release are located in the NAc; thus, our withdrawal studies focused on the NAc, where the actual release of neurotransmitter occurs. While MLA did not precipitate any change in

CaMKII or synpasin I function in the NAc, DHβE precipitated a significant decrease in CaMKII and synapsin I activity and protein level at doses that precipitated aversion in our CPA model (Chapter 2). To complement these pharmacological studies, we measured CaMKII and synapsin I function in the NAc from β2 and α7 KO mice and corresponding WT littermates after treatment with mecamylamine (2 mg/kg, s.c.) at a dose that precipitated withdrawal signs in nicotine-dependent mice (Chapters 2 and 3; Damaj et al., 2003). There was a significant decrease in CaMKII and synapsin I activity after treatment with mecamylamine in chronic nicotine infused WT mice; however, this decrease was absent in β2 KO mice. Conversely, a decrease in CaMKII and synapsin I activity was observed in α7 KO mice after mecamylamine precipitated withdrawal. While DHβE precipitated a significant decrease in CaMKII and synapsin I total protein level, there was no change in the level of either protein after mecamylamine treatment. The phosphorylated, or activated, state of the protein is an indication of protein activity, while the protein level reflects the total expression of protein in the system. Both DHβE and mecamylamine decreased protein activity; however, the differences in protein level are difficult to interpret. Mecamylamine in general is a non-selective nAChR antagonist (Papke et al., 2001), and has less selectivity for α4β2* nAChRs than DHβE; however, we are unable to explain the differences in total protein level that occurred after treatment with the different antagonists. Regardless, the results still support our data suggesting a role for β2* nAChRs in nicotine withdrawal-induced decreases in CaMKII and synapsin I activity. Because our chronic nicotine assessment revealed a significant increase in p603 expression, this rules against the possibility that our withdrawal data are the result of chronic nicotine exposure rather than

withdrawal. Although an examination of CaMKII was not provided, because p603 is the site specifically phosphorylated by CaMKII, we expect that there is also an increase in pCaMKII expression following chronic nicotine exposure. Together, our biochemical studies using pharmacological agents and the complementary transgenic mouse approach suggest that β2-containing, but not α7* nAChRs are involved in nicotine induced changes in CaMKII and synapsin I function in the VTA and NAc after acute nicotine, and in the NAc after nicotine withdrawal.

The α6β2* and α4α6β2* nAChR subtypes are located on presynaptic DA and GABAergic neurons in the VTA and are involved in nicotine-stimulated DA release in the striatum (Champtiaux et al., 2003; Salminen et al., 2004; Lai et al., 2005). Many studies support the vital role of the neurotransmitter DA in nicotine dependence. The nicotine induced increase in VTA DA neuron firing rate (Grenhoff et al., 1986), and subsequent DA release in the NAc is a process thought to underlie the addictive properties of nicotine (Ponteiri et al., 1996). Alternatively, studies report decreased DA neuronal activity in the VTA (Liu and Jin, 2004) and decreased DA output in the NAc after nicotine withdrawal (Hildebrand et al., 1998; Rada et al., 2001). Indeed, it is hypothesized that this relative deficiency in DA release following cessation of nicotine exposure accounts for many of the mood disorders, craving, and anhedonia that persist in many smokers long after quitting (Benowitz, 2008). Because CaMKII and synapsin I are essential for neurotransmitter release, these two proteins are sufficient biochemical markers of neurotransmitter release. The decrease in CaMKII and synapsin I function in the NAc after nicotine withdrawal may

correspond to the decrease in DA release observed in the NAc after nicotine withdrawal. Our behavioral pharmacological assessment in Chapter 5 revealed that the CaMKII inhibitor, KN-93, significantly enhanced the anxiety-related response and showed a strong trend for enhancement of nicotine CPA, also suggesting that CaMKII is decreased after nicotine withdrawal. Further, our behavioral studies in Chapters 2 and 3 revealed a role for β2-containing receptors, specifically α6β2* and/or α4α6β2* nAChR subtypes in affective nicotine withdrawal behaviors. Although with the current results, we cannot make a direct correlation between our behavioral and biochemical studies, these initial results imply that β2-containing nAChRs, which are critical in affective nicotine withdrawal, also mediate the nicotine withdrawal induced decreases in CaMKII and synapsin I function in the NAc.

GENERAL DISCUSSION

A. Rationale and Summary of Overall Hypothesis

In the United States, tobacco dependence is the leading cause of preventable mortality. While the positive rewarding and reinforcing effects of nicotine motivate continued smoking behavior (Kenny and Markou, 2001), many studies suggest that avoidance of the negative emotional state produced by nicotine withdrawal represents a motivational component that promotes continued tobacco use and relapse after smoking cessation. Indeed, the most commonly reported reason for relapsing into smoking during quit attempts is the desire to relieve the discomforts of smoking withdrawal (US Department of Health and Human Sciences, 1988). Although there are smoking cessation therapies available, the success rate is modest, as only 20-25% of smokers remain abstinent after one year (Gonzales et al., 2006). Because smoking is such a widespread health problem, it is important to understand the molecular and behavioral mechanisms of nicotine withdrawal to generate more effective smoking cessation therapies.

Despite the important contribution of withdrawal symptoms to the maintenance of tobacco use, few studies assess the negative motivational component associated with nicotine withdrawal. Currently, due to the low selectivity of most available nAChR antagonists, the identity of nAChR subtypes involved in nicotine withdrawal remains unclear. Furthermore, studies which address the signaling processes underlying nicotine withdrawal are lacking; therefore, our studies focused on elucidating the receptor and post-receptor mediated mechanisms involved in nicotine withdrawal. To address this aim, our

studies focused on three areas of research. First, we identified the major nAChR subtypes involved in affective and physical nicotine withdrawal behaviors. Because of the importance of affective signs, we measured two different affective nicotine withdrawal behaviors; anxiety-related behavior and aversion. Because these signs involve different brain regions, with the dorsal raphé nucleus being crucial in mediating nicotine's effects on anxiety (Cheeta et al., 2001), and the basolateral amygdala being involved in aversion (Zanoveli et al., 2007), it is possible that they may involve different nAChR subtypes. Second, we evaluated the role of important Ca^{2+}-dependent mechanisms, such as L-type VGCCs and CaMKII, in nicotine withdrawal behaviors. Third, we conducted initial biochemical studies to aid in elucidating the relationship between the altered behavioral responses *in vivo* and the *in vitro* receptor-mediated Ca^{2+}-dependent mechanisms involved in nicotine withdrawal. Overall, we hypothesized that major nAChR subtypes have differential roles in the nicotine withdrawal syndrome. Specifically, the α4α6β2* nAChR subtype is involved in affective nicotine withdrawal behaviors. This subtype also mediates nicotine-withdrawal induced decreases in CaMKII function in the NAc. The decrease in CaMKII function leads to decreased phosphorylation of CaMKII substrates, specifically synapsin I, a protein essential for regulation of neurotransmitter release.

B. Nicotinic receptor subtypes have differential roles in nicotine withdrawal

The α4α6β2* nAChR subtype is specifically involved in affective nicotine withdrawal

Using spontaneous, precipitated, and CPA models adapted for mouse studies, we showed that major nAChR subtypes have differential roles in nicotine withdrawal. We began our study with the α4β2* nAChR subtype, a main nAChR subtype in the brain that is located on presynaptic dopaminergic and GABAergic neurons in the mesocorticolimbic drug pathway. In β2 KO mice, there was a loss of anxiety-related behavior and aversion in the CPA model compared to WT mice, but normal withdrawal-induced somatic signs and hyperalgesia (Figures 8, 9, and 12). These results were complemented using the β2-selective antagonist, DHβE, which precipitated significant aversion in the CPA model (Fig. 3). Previous studies from our lab also showed that DHβE precipitated anxiety-related behavior, but not somatic signs or hyperalgesia in chronic nicotine infused mice (Damaj et al., 2003). Immunoprecipitation and ligand-binding studies have also shown that the α5 and α6 subunits are coexpressed with α4 and β2 subunits on midbrain dopaminergic neurons and terminals (Le Novère et al., 1996; Klink et al., 2001; Champtiaux et al., 2002; Zoli et al., 2002); therefore, we also evaluated the role of α5 and α6 nAChR subunits in nicotine withdrawal. Central administration of the α6-selective antagonist, MII[H9A;L15A], dose-dependently blocked the expression of the anxiety-related response and CPA in nicotine withdrawn mice, but had no effect on somatic signs or the hyperalgesia response (Figures 11 and 13). Additionally, the α6/α3β2* selective antagonist, MII, blocked the expression of anxiety-related behavior (Fig. 16), but not CPA

(Fig. 19), suggesting a role for the α6β2* subtype in the anxiety-related response. Conversely, α5 KO mice displayed significant anxiety-related behavior, CPA, and hyperalgesia, but a reduction in total somatic signs (Figures 10 and 12). Although α6β2*, α4α6β2*, and α4α5β2* subtypes are found in the mesocorticolimbic drug pathway and are involved in striatal DA release (Champtiaux et al., 2003; Salminen et al., 2004; Lai et al., 2005), the results suggest that the α6β2* and/or α4α6β2*, but not the α4α5β2* nAChR subtype, are specifically involved in affective nicotine withdrawal behaviors. Together with previous studies, these data suggest that α6β2* and/ or α4α6β2* nAChR subtypes are critical for affective nicotine withdrawal.

The α7 subtype is involved in the nicotine withdrawal-induced hyperalgesia response

The homomeric α7 nAChR is also a major subtype found on neurons in the mesocorticolimbic drug pathway and has been implicated in physical withdrawal behaviors. In fact, in our studies, mice null for the α7 subunit displayed a loss of nicotine withdrawal-induced hyperalgesia, but significant anxiety-related behavior, CPA, and somatic signs (Figures 14, 15 and 18). A previous α7 nAChR KO study from our laboratory using the oral route of chronic nicotine administration and the spontaneous nicotine withdrawal model produced similar findings, showing a loss of hyperalgesia in α7 KO mice, but otherwise normal somatic signs and anxiety-related response (Grabus et al., 2005). We also found that MLA, an α7 antagonist, did not precipitate aversion in the CPA model in chronic nicotine infused mice, thus, complementing our results using transgenic animals (Fig. 3). MLA was also shown to precipitate a significant hyperalgesia response in

nicotine dependent mice (Damaj et al., 2003). Overall, these results implicate a role for α7 nAChRs in some aspects of physical withdrawal, but not affective nicotine withdrawal. The data also suggests that our two physical measures of nicotine withdrawal (somatic signs and hyperalgesia), are mediated by different nAChR subtype populations. Although α7 KO mice showed a loss of the hyperalgesia response, somatic signs were still present. Hyperalgesia is measured as a physical sign in the mouse; however, studies have suggested that spinal and supraspinal nAChR populations mediate the hyperalgesia response (Schmidt et al., 2001; Damaj et al., 2002). Our hyperalgesia measure was conducted using the hot plate, which measures supraspinal mechanisms; thus, we cannot rule out the possibility that the nicotine withdrawal induced hyperalgesia response is mediated by neuronal α7 nAChRs, which do not contribute to the centrally mediated component of somatic nicotine withdrawal. It is also noted that MLA precipitated mild somatic signs of withdrawal in WT mice (Damaj et al., 2003). MLA can antagonize α6, α3, and β3 nAChR subunits at doses typically used to block α7 nAChRs (Mogg et al., 2002). Indeed, it was revealed that MLA precipitated significant somatic signs in nicotine dependent α7 KO mice (Salas et al., 2007). Our studies using α3 nAChR antagonists revealed a role for α3-containing nAChRs in somatic nicotine withdrawal signs, as will be discussed in the next section. Conversely, α6-containing receptors are not involved in somatic nicotine withdrawal signs; thus, it is possible that the observed behavioral responses were attributed to effects on other nAChR subunits, such as the α3 and/or β3 subunits.

α3-containing nAChRs are involved in physical aspects of nicotine withdrawal

The α3 subunit is coexpressed with α5 and β4 nAChRs in the peripheral ganglia, MHb and IPN (Wada et al., 1990; Zoli et al., 1995; Quick et al., 1999; Whiteaker et al., 2002). The α3 nAChR subunit is also expressed in brain areas that have been implicated as having a role in nicotine dependence behaviors, and can coassemble with β2 nAChRs to form functional receptors on DA neurons and terminals (Le Novère and Changeux, 1995; Le Novère et al., 1996; Klink et al., 2001). It was shown that β4 KO mice do not display somatic signs after nicotine withdrawal (Salas et al., 2004), suggesting a role for the β4 subunit in somatic nicotine withdrawal. Additionally, our data suggested a role for the α5 subunit in somatic nicotine withdrawal, as α5 KO mice displayed a significant reduction in total somatic signs. It was also shown recently that a common haplotype in the CHRNA5/CHRNA3/CHRNB4 gene cluster, which codes for the α5, α3, and β4 subunits respectively, predisposes to nicotine dependence (Berrettini et al., 2008); therefore, we identified a role for α3-containing receptors in nicotine withdrawal behaviors using MII and AuIB, selective α3β2* and α3β4* nAChR subtype antagonists respectively.

AuIB dose-dependently attenuated somatic nicotine withdrawal signs, but had no effect on expression of the anxiety-related response, hyperalgesia, or CPA (Figures 17 and 19), suggesting a role for the α3β4* nAChRs in somatic, but not affective nicotine withdrawal. Results also show that the α3β2* selective antagonist, MII, dose-dependently blocked expression of the anxiety-related response and attenuated somatic signs (Fig. 16). MII had no effect on the hyperalgesia response at any dose tested (Fig. 16). Surprisingly,

MII did not block expression of CPA, even after doubling the dose used to block the anxiety-related response (Fig. 19). Taken together, these results also stress and important role for β2*, but not β4* nAChRs, in affective nicotine withdrawal. Studies suggest that MII may not discriminate well between α3 and α6 nAChRs (Kuryatov et al., 2000), as the α6 and α3 nAChR subunits share considerable sequence homology (Le Novère and Changeux, 1995). The MII analog and selective α6 antagonist, MII[H9A;L15A], effectively discriminates between α3 and α6 nAChR subunits (McIntosh et al., 2004). Our results from Chapter 3 showed a role for α6-containing nAChRs in nicotine CPA and the anxiety-related response, but not physical withdrawal, suggesting that α6-containing nAChRs, and not α3β2* nAChRs, are important for nicotine CPA, while both α6β2* and α3β2* nAChRs are involved in the withdrawal-induced anxiety-related response. Indeed, anxiety-related behavior and CPA are mediated by two different brain regions; the dorsal raphé nucleus (Cheeta et al., 2001) and the basolateral amygdala (Zanoveli et al., 2007) respectively; thus, it is possible that the two behaviors are mediated by different subtypes. The dorsal raphé nucleus also receives innervations from the MHb (Morris et al., 1999), a brain area rich in α3 nAChR expression (Quick et al., 1999); thus, the MHb may also be involved in anxiety-related behaviors. Conversley, because MII does discriminate well between α6 and α3 nAChR subunits, we cannot rule out the possibility that the observed MII effect in the plus maze test are attributed to actions at α6β2* nAChRs rather than α3β2*.

These results also implicate the importance of receptor subtype composition. While our data showed that β2-containing nAChRs were not involved in somatic nicotine withdrawal, because MII attenuated somatic withdrawal signs, it cannot be ruled out that the α3β2* subtype is involved in somatic nicotine withdrawal signs. Additionally, it is noted that α3β2* and α3β4* nAChRs are involved in somatic signs, but not the hyperalgesia response, whereas the α7 subtype was shown to be involved in the hyperalgesia response, but not somatic signs. These results are consistent with our proposal that the physical withdrawal signs measured in our model (somatic signs and hyperalgesia) are mediated by different nAChR subtypes. It is also possible that the brain regions in which these nAChR subtypes are expressed can influence their effects.

The α5, α3, and β4 nAChR subunits coassemble in peripheral ganglia, MHb and IPN (Wada et al., 1990; Zoli et al., 1995; Quick et al., 1999; Whiteaker et al., 2002), and have all been shown to be involved in somatic nicotine withdrawal. Our studies using central administration of AuIB, a selective α3β4* antagonist, support a role for central α3β4* nAChRs in somatic nicotine withdrawal signs. Interestingly, somatic nicotine withdrawal signs are mediated by both central and peripheral nAChR populations (Watkins et al., 2000); thus, we propose that α5 and α3β4* nAChRs contribute to expression of somatic signs through location in the peripheral ganglia, as well as centrally in the MHb and IPN. The MHb and IPN have been implicated in behaviors associated with drug dependence. A synthetic derivative of the African shrub ibogaine, 18-Methoxyroconaridine (18-MC), was shown to be an inhibitor of α3β4* nAChRs *in vitro* (Glick et al., 2002). When administered

into the MHb and IPN, 18-MC blocked morphine and nicotine self-administration (Glick et al., 2000; Glick et al., 2006), somatic morphine withdrawal signs (Panchal et al., 2005), and dopamine sensitization to morphine (Taraschenko et al., 2007) in rats. Although the available studies do not provide direct evidence of a α3α5β4* subtype composition in the brain, studies indicate that α3, α5, and β4 subunit mRNA was present in nearly 100% of neurons isolated from rat MHb (Sheffield et al., 2000). Overall, the data suggests that α3α5β4* gene clusters located in the MHb and in peripheral ganglia are important in somatic nicotine withdrawal.

This extensive investigation of the identity of nAChR subtypes involved in various aspects of nicotine withdrawal yielded important findings. A pictorial depiction of the proposed subtypes involved in physical and affective nicotine withdrawal is presented in Figure 44.

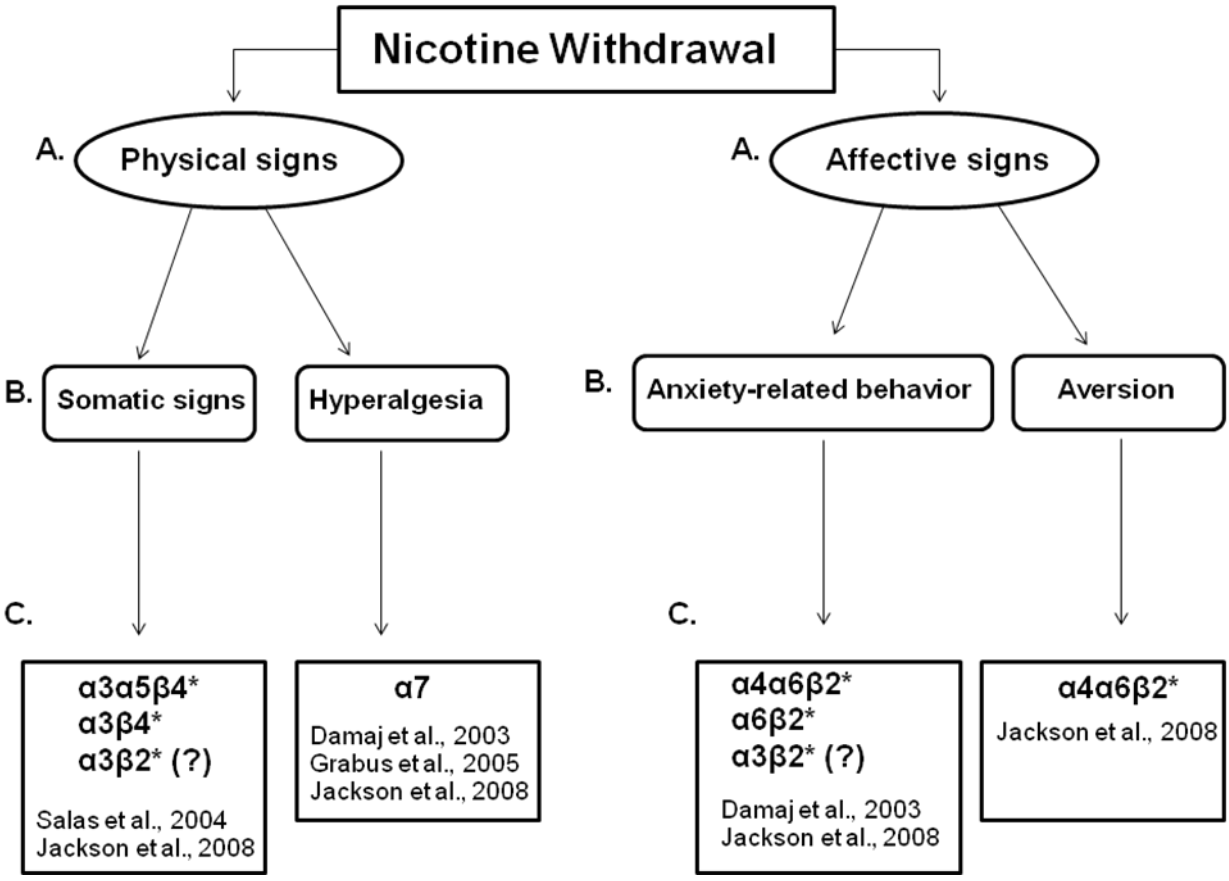


Figure 44. Overall depiction of the role of major nAChR subtypes in physical and affective nicotine withdrawal signs as revealed in our studies and others. A. Represents the two aspects of the nicotine withdrawal syndrome. B. Representation of the specific signs categorized under each aspect of nicotine withdrawal that were measured in the current studies. C. The major nAChR subtypes that were found to be involved in the various aspects of nicotine withdrawal. In addition to the current studies, citations of studies that support our conclusions for the subtypes' role in nicotine withdrawal are listed.

C. L-type VGCCs and CaMKII differentially regulate nicotine withdrawal

Studies have suggested a role for L-type VGCC and CaMKII in the effects of several drugs of abuse, including morphine, cocaine, amphetamine, and nicotine (Tan, 2002; Wang Z et al., 2003; Licata et al., 2004; Biala and Weglinska, 2005). Although the current studies evaluating Ca^{2+}-dependent mechanisms in nicotine dependence provide evidence of a role for Ca^{2+} signaling, studies assessing the affective component of nicotine withdrawal are lacking. There is also no available behavioral correlation for the biochemical studies which evaluate nicotine-induced Ca^{2+} mechanisms *in vitro*; thus, the relevance of these mechanisms to nicotine withdrawal is unclear. The study by Biala and Weglinska (2005) found that L-type VGCC blockers attenuated somatic nicotine withdrawal signs; however, the study utilized high doses of L-type VGCC blockers, which our studies found significantly reduced locomotor activity (Fig. 20). Furthermore, such high doses may inhibit α3* and α7* nAChR-mediated currents (Wheeler et al., 2006).

In our study, L-type VGCC blockers attenuated somatic signs and hyperalgesia in nicotine-withdrawn mice, but there was no effect on anxiety-related behavior or development of CPA (Figures 21 and 23). Because L-type VGCC blockers have been shown to inhibit α3* and α7* nAChRs *in vitro* (Wheeler et al., 2006), we used an L-type VGCC activator, (±)Bay K8644, to complement our results. (±)Bay K8644 enhanced somatic nicotine withdrawal signs and the hyperalgesia response (Fig. 22). Taken together, our studies support a role for L-type VGCCs in physical, but not affective nicotine withdrawal. Clinically, this may suggest that L-type VGCC blockers would not be very

successful in alleviating withdrawal symptoms in abstinent smokers. Although somatic signs do contribute to the discomfort associated with the nicotine withdrawal syndrome, the affective signs are suggested to be of greater motivational significance in contributing to relapse (Koob et al., 1993; Markou et al., 1998).

While the L-type VGCC studies yielded clear results, the role of CaMKII in nicotine withdrawal behaviors appears more complex. While pharmacological blockade of CaMKII using the inhibitor KN93 dose-dependently attenuated somatic nicotine withdrawal signs, it enhanced the affective component of nicotine withdrawal (Figures 24 and 28). To examine this effect further, we treated chronic nicotine infused mice with KN93 and found that the inhibitor precipitated anxiety-related behavior, but had no effect on somatic signs or hyperalgesia (Fig. 25). These pharmacological results suggest that CaMKII has opposing roles in nicotine withdrawal. While KN93 enhanced the anxiety-related response, suggesting that CaMKII activity is decreased after nicotine withdrawal, KN93 attenuated somatic nicotine withdrawal signs, suggesting that CaMKII activity is increased after nicotine withdrawal. One explanation for this effect is that CaMKII function after nicotine withdrawal differs depending on the brain region and nAChR subtype involved. We proposed that affective withdrawal signs involve α4α6β2* nAChR subtypes in the VTA, while somatic nicotine withdrawal involves the α3α5β4* subtype in the MHb. After chronic antidepressant treatment, CaMKII activity differed based on the brain region and presynaptic compartment (i.e. synaptic vesicles vs. synaptic membranes) (Schneider et al., 2007). Additionally, our biochemical studies in Chapter 6 showed that

acute nicotine-induced changes in CaMKII function in the VTA are nicotinic receptor mediated effect (Fig. 30). Therefore, because we observed attenuation of somatic signs after KN93 treatment, it is possible that there is an increase in CaMKII function after nicotine withdrawal in the MHb that is mediated by α3α5β4* nAChRs. The enhanced anxiety-related response after KN-93 treatment and the trend observed in the CPA model suggest a decrease in CaMKII function after nicotine withdrawal, which may be mediated by α4α6β2* nAChR subtypes in the NAc and/or VTA.

Conversely, our transgenic assessment revealed an opposite role for CaMKII than that observed in our pharmacological assessment. Saline infused CaMKII HT mice had decreased baseline anxiety levels on the plus maze compared to WT counterparts, consistent with CaMKII mutants having increased exploratory behavior in the open field and Y-maze (Silva et al., 1992b), and there was a loss of anxiety-related behavior in nicotine-infused CaMKII HTs after mecamylamine treatment (Fig. 26). These results suggest that CaMKII activity is increased after nicotine withdrawal. There was no significant difference in somatic signs, hyperalgesia, or aversion in the CPA model between saline and nicotine infused mice (Figures 26 and 28). Several possible explanations for this discrepancy were discussed in Chapter 5. Overall, the results suggest that L-type VGCC and CaMKII are differentially regulated in nicotine withdrawal, although CaMKII, which was shown to be involved in both somatic and affective nicotine withdrawal, may be a more clinically relevant target.

Depression is an affective symptom associated with nicotine withdrawal. Indeed, the antidepressant, bupropion, is clinically approved for smoking cessation and has been proposed to act via promoting dopaminergic function (Nestler and Carlezon, 2006). Changes in synaptic plasticity are involved in the pathophysiology of depression and in the mechanism of antidepressants (Manji et al., 2001). CaMKII, being a kinase involved in synaptic plasticity, has been shown to be a target of antidepressants. Reduced α-CaMKII expression was found in the brains of subjects with bipolar and unipolar depression (Xing et al., 2002), and antidepressants activate CaMKII in hippocampal neuronal cell bodies by phosphorylation of Thr286 (Tiraboschi et al., 2004). An inhibitor of CaMKII exacerbated anxiety-related behavior in our studies, suggesting decreased CaMKII levels after nicotine withdrawal. Because antidepressants were shown to enhance CaMKII, it is possible that CaMKII is a relevant target in alleviating the depression and overall negative emotional state associated with nicotine withdrawal. Additionally, we cannot rule out the possibility that CaMKIV is involved in these effects, as KN93 also inhibits this kinase (Enslen et al., 1994). Suppression of CaMKIV in the NAc increased anxiety-related behavior in rats (Schneider et al., 2007), suggesting that CaMKIV in the NAc is involved in mechanisms associated with anxiety. The effects of nicotine on CaMKIV to date have not been assessed; however, CREB, a downstream target of CaMKIV, was shown to be involved in mechanisms associated with nicotine dependence, including reward (Walters et al., 2005) and withdrawal (Pandey et al.,2001; Brunzell et al., 2003; Pluzarev and Pandey, 2004). Further, disruption of CREB function in the NAc is also associated with increased anxiety responses, while overexpression of CREB was shown to be anxiolytic (Barrot et al., 2002;

Barrot et al., 2005). Taken together, we propose that intracellular Ca^{2+}-dependent kinases, such as CaMKII and CaMKIV, are involved in the affective component of nicotine withdrawal, and are critical targets for the alleviation of affective symptoms associated with nicotine withdrawal. Additionally, the NAc may be a target brain area involved in these emotional responses.

D. Nicotine withdrawal-induced decreases in CaMKII and synapsin I function are mediated through β2-containing nAChRs

Studies suggest a role for CaMKII and synapsin I in various drugs of abuse such as cocaine, morphine, and amphetamine (Matus-Leibovitch et al., 1995; Iwata et al., 1996; Iwata et al., 1997a; Iwata et al., 1997b; Tan, 2002; Licata et al., 2004; Wang et al., 2003). Additionally, our behavioral studies showed that Ca^{2+}-dependent mechanisms, such as L-type VGCC and CaMKII, are involved in nicotine withdrawal behaviors; therefore, we wanted to determine the relationship between these nicotine withdrawal-induced behavioral modifications and the *in vitro* Ca^{2+}-dependent molecular mechanisms involved in nicotine withdrawal. We measured CaMKII and synapsin I activity in the VTA and NAc after acute systemic nicotine administration and found that nicotine induced increases in the activity of both proteins, and these increases are mediated through β2-containing nAChRs (Figures 29; 31-34). Further, we measured activity and protein level in the NAc after chronic nicotine and nicotine withdrawal, as release of neurotransmitter and the presynaptic nAChRs that mediate neurotransmitter release are located in this brain region. Synapsin I activity was increased in the NAc after chronic nicotine (Fig. 35), suggesting

that tolerance does not develop to the acute nicotine-induced increase in this protein. Results from the nicotine withdrawal assessment show that DHβE, but not MLA, precipitate significant decreases in CaMKII and synapsin I activity and level at doses that precipitate significant aversion in the CPA model (Figures 3; 36-39). Our studies using transgenic animals complemented this data, as the mecamylamine-precipitated decrease in CaMKII and synapsin I activity in the NAc was present in α7 KO, but not in β KO mice (Figures 41-43). Taken together, these results suggest that β2*, but not α7*, nAChRs mediate the acute nicotine-induced increase in CaMKII and synapsin I activity in the VTA and NAc, as well as the nicotine withdrawal-induced decrease in activity and level in the NAc.

The α6β2* and α4α6β2* nAChR subtypes are located on presynaptic DA and GABAergic neurons in the VTA and are involved in nicotine-stimulated DA release in the striatum (Champtiaux et al., 2003; Salminen et al., 2004; Lai et al., 2005). Indeed, the nicotine induced increase in VTA DA neuron firing rate (Grenhoff et al., 1986), and subsequent DA release in the NAc is a process thought to underlie the addictive properties of nicotine (Ponteiri et al., 1996). Alternatively, studies report decreased DA output in the NAc after nicotine withdrawal (Hildebrand et al., 1998; Rada et al., 2001). It is hypothesized that this relative deficiency in DA release following cessation of nicotine exposure accounts for many of the mood disorders, craving, and anhedonia that persist in many smokers long after quitting (Benowitz, 2008). CaMKII and synapsin I are sufficient biochemical markers of neurotransmitter release, as these proteins are essential for

neurotransmitter release. The decrease in CaMKII and synapsin I function in the NAc after nicotine withdrawal may correspond to the decrease in DA release observed in the NAc after nicotine withdrawal. Our behavioral pharmacological assessment in Chapter 5 revealed that the CaMKII inhibitor, KN93, significantly enhanced the anxiety-related response and showed a strong trend for enhancement of nicotine CPA, which suggests that CaMKII is decreased after nicotine withdrawal (Figures 24 and 28). Further, our behavioral studies in Chapters 2 and 3 revealed a role for β2-containing receptors, specifically α6β2* and/or α4α6β2* nAChR subtypes in affective nicotine withdrawal behaviors (Figures 3, 8, 9, 11-13).

Overall, these initial results show that CaMKII and synapsin I activity in the NAc are decreased after nicotine withdrawal, and these decreases are mediated through β2-containing nAChRs. Although we did not test the α6 antagonists, MII and MII [H9A;L15A], or the α3 antagonists, MII and AuIB, in our *in vitro* studies, we propose that the α6β2* and /or α4α6β2* subtypes, and not α3* or α7* containing nAChRs, mediate the nicotine withdrawal-induced decreases in CaMKII and synapsin I function in the NAc. As mentioned, these subtypes are presynaptically located on DA terminals in the striatum and are involved in DA release in this brain region. Further, MLA, an α7 antagonist, did not precipitate a decrease in CaMKII or synapsin I function, and the decrease in activity was present in α7 KO mice. Our behavioral studies implicate α7 and α3-containing nAChRs in physical nicotine withdrawal (Chapter 3), yet we saw no effect with MLA at doses that precipitated withdrawal in mice (Damaj et al., 2003). Based on this data, we propose that

while α7 and α3-containing nAChRs are involved in physical nicotine withdrawal signs, they are not relevant to the molecular changes that occur after nicotine withdrawal. It is noted that the L-type VGCC blocker nimodipine, also blocked the acute nicotine induced increase in CaMKII activity in the VTA (Fig. 30); however, because we did not evaluate the role of L-type VGCC in our biochemical assessment after nicotine withdrawal, we cannot determine if this effect is relevant to the decrease in protein activity after nicotine withdrawal. Additional studies using behaviorally relevant nimodipine doses (1-2 mg/kg, i.p.) are necessary to further evaluate this effect.

E. Conclusions and Implications

Overall, our research contributes to the understanding of the behavioral and molecular mechanisms underlying nicotine withdrawal. These studies identified major nAChR subtypes, post-receptor Ca^{2+}-dependent mechanisms, and brain regions important in nicotine withdrawal. Further, by demonstrating that the decreases in CaMKII and synapsin I function, which may contribute to the decreased DA release observed after nicotine withdrawal, are mediated through β2-containing nAChRs, which are crucial for expression of affective nicotine withdrawal signs, this study stresses the importance of affective nicotine withdrawal as a motivational component of relapse in smoking behavior. Studies suggest that although somatic signs are an important component of the nicotine withdrawal syndrome, the affective signs are of greater motivational significance in contributing to relapse (Koob et al., 1993; Markou et al., 1998). As mentioned, it is also hypothesized that this relative deficiency in DA release following cessation of nicotine

exposure accounts for many of the mood disorders, craving, and anhedonia that persist in many smokers long after quitting (Benowitz, 2008). Indeed, reduced α-CaMKII expression was found in the brains of subjects with bipolar and unipolar depression (Xing et al., 2002), and anti-depressants have been shown to increase CaMKII phosphorylation in hippocampal neuronal cell bodies (Tiraboschi et al., 2004). Further, the anti-depressant, bupropion, which blocks nAChRs (Slemmer et al., 2000), and inhibits the DA transporter, thus enhancing DA function (Nestler and Carlezon, 2006), is clinically used as a smoking cessation therapy. Taken together, these studies suggest that presynaptic β2-containing nAChRs, specifically α6β2* and/or α4α6β2* subtypes, which mediate striatal DA release, CaMKII, and synapsin I, are critical components of the affective nicotine withdrawal syndrome, and may be clinically relevant to smoking cessation; thus, more research should focus on the role of these targets in development of nicotine dependence. Overall, the research in this dissertation contributes to the understanding of the behavioral and molecular mechanisms of nicotine withdrawal, and provides new insight into potential targets for more effective smoking cessation therapies.

F. Future Studies

A major part of our effort during the Ph.D. work was dedicated to identifying the major nAChR subtypes involved in nicotine withdrawal. Using pharmacological methods and genetically modified mice, we showed that the various aspects of nicotine withdrawal are mediated by different nAChR subtypes. While we were able to identify the major subtypes involved (Fig. 44), more studies are needed to identify the exact composition of

these subtypes and important brain regions involved in nicotine withdrawal behaviors. To support our proposal for a role of the α3α5β4* subtype in somatic nicotine withdrawal signs, future studies include testing the α3β4* antagonist AuIB in α5 KO mice. Our studies showed a significant reduction in total somatic signs in α5 KO mice and a dose-dependent attenuation of somatic signs after central administration of AuIB. We will determine the role of the α3α5β4* subtype by determining if we can attenuate somatic signs in α5 KO mice with AuIB. This would also further implicate the MHb as a brain region involved in manifestation of somatic signs.

Additionally, our results show that β2-containing nAChRs mediate affective nicotine withdrawal signs. Because the α4β2* subtype is the most abundant subtype in the brain, we proposed that this subtype is involved in affective nicotine withdrawal behaviors. Our pharmacological data using the α6 and α6/α3β2* antagonists, MII[H9A;L15A] and MII respectively, suggest a role for α6β2* and/or α4α6β2* nAChRs in affective nicotine withdrawal. To further evaluate these results, we will obtain and breed α4 and α6 KO mice to test in our nicotine withdrawal models. This complementary approach will allow us to further differentiate between the importance of α6β2* and α4α6β2* subtypes in affective nicotine withdrawal.

Another major finding of our work was the involvement of L-type VGCCs and CaMKII in nicotine withdrawal behaviors. Indeed, results from our second specific aim clearly showed a role for L-type VGCCs in physical nicotine withdrawal, but not affective withdrawal. CaMKII results were more difficult to interpret due to differences between our

pharmacological and transgenic mouse data. We used CaMKII HT mice in our experiments because of our failed attempts to generate CaMKII KO mice, as CaMKII HT females do not care for their pups. To alleviate this problem, we will attempt to foster pups born from CaMKII HT mothers in order to obtain CaMKII KOs. Another option would be the use of CaMKII conditional KOs, which can be obtained from Jackson Laboratories.

Although our data from specific aim two do suggest a role for Ca^{2+}-dependent mechanisms in nicotine withdrawal, we used another strategy to explore the involvement of these mechanisms. We examined the relationship between the altered behavioral responses *in vivo* and the *in vitro* receptor-mediated Ca^{2+}-dependent mechanisms involved in nicotine withdrawal. For our third specific aim, results showed that there are decreases in CaMKII and synapsin I activity after nicotine withdrawal, and these decreases are mediated through β2-containing nAChRs. We propose that the nicotine withdrawal-induced decreases are mediated through α4β2* and/or α4α6β2* nAChR subtypes, which are involved in affective nicotine withdrawal signs. To further examine this effect, we will evaluate CaMKII and synapsin I activity and level in the NAc of α4 and α6 KO mice after nicotine withdrawal. We complement the α6 KO mouse studies using the α6 antagonist, MII[H9A;L15A], and the α6/α3β2* antagonist, MII. Additionally, we propose that the α3β4* and/or α3α5β4* nAChR subtypes, which mediate somatic nicotine withdrawal signs, are not involved in the nicotine withdrawal induced decrease in CaMKII and synapsin I; therefore, we will also measure CaMKII and synapsin I activity and level in the NAc in α5 KO mice, and in mice after treatment with the α3β4* antagonist, AuIB. To

correlate the decrease in CaMKII and synapsin I activity with our behavioral results, we will complete a time course of the biochemical changes and compare them to our spontaneous withdrawal assessment. We hypothesize that the decrease in CaMKII and synapsin I activity is relevant to affective nicotine withdrawal behaviors; therefore, we expect to see a decrease in CaMKII and synapsin I activity and level in the NAc after spontaneous nicotine withdrawal that correlates with the increase in severity of affective withdrawal signs that occurs after the first few days after spontaneous nicotine withdrawal (Damaj et al., 2003). As the severity of the withdrawal syndrome peaks and subsides over the course of several days, we expect that activity and protein level of CaMKII and synapsin I will return to baseline levels.

These proposed studies will further support our hypothesis of the importance of the α6β2* and/or α4α6β2* subtypes and the decrease in CaMKII and synapsin I function in the NAc in the manifestation of affective nicotine withdrawal behaviors.

Literature Cited

Literature Cited

Anderson SM, Famous KR, Sadri-Vakili G, Kumaresan V, Schmidt HD, Bass CE, Terwilliger EF, J Cha J, Pierce RC (2008) CaMKII:a biochemical bridge linking accumbens dopamine and glutamate systems in cocaine seeking. Nat Neurosci 11:344-353.

Antkiewicz-Michaluk L, Michaluk J, Romanska I, Vetulani J (1990) Corticol dihydropyridine binding sites and behavioral syndrome in morphine-abstinent rats. Eur J Pharmacol 180:129-135.

Allen SS, Hatsukami D, Christianson D, Brown S (2000) Effects of transdermal nicotine on craving, withdrawal and premenstrual symptomatology on short-term smoking abstinence during different phases of the menstrual cycle. Nicot Tob Res 2:231-241.

American Psychiatric Association (1994) Diagnostic and statistical manual of mental disorders, 4th ed. American Psychiatric Press, Washington, DC.

Balfour DJ and Fagerstrom KO (1996) Pharmacology of nicotine and its therapeutic use in smoking cessation and neurodegenerative disorders. Pharmacol Ther 72:51-81.

Bardo MT, Green TA, Crooks PA, Dwoskin LP (1999) Nornicotine is self-administered intravenously by rats. Psychopharmacology 146:290-296.

Barrott M, Olivier JD, Perrotti LI, DiLeone RJ, Berton O, Eisch AJ, Impey S et al. (2002) CREB activity in the nucleus accumbens shell controls gating of behavioral responses to emotional stimuli. Proc Natl Acad Sci USA 99:11435-11440.

Barrott M, Wallace DL, Bolanos CA, Graham DL, Perrotti LI, Neve RL, Chambliss H, Yin JC, Nestler EJ (2005) Regulation of anxiety and initiation of sexual behavior by CREB in the nucleus accumbens. Proc Natl Acad Sci USA 102:8235-8242.

Benowitz NL (2008) Neurobiology of nicotine addiction: implications for smoking cessation treatment. Am J Med 121:3-10.Review.

Benwell ME, Balfour DJ, Anderson JM (1988) Evidence that tobacco smoking increases the density of (-)-[^{3}H]nicotine binding sites in human brain. J Neurochem 50:1243-1247.

Benwell ME, Balfour MJ (1992) The effects of acute and repeated nicotine treatment on nucleus accumbens dopamine and locomotor activity. Br J Pharmacol 105:849-856.

Berke JD and Hyman SE (2000) Addiction, dopamine, and the molecular mechanisms of memory. Neuron 25:515-532.

Berrettini W, Yuan X, Tozzi F, Song K, Francks C, Chilcoat H, Waterworth D, Muglia P, Mooser V (2008) α-5/α-3 nicotinic receptor subunit alleles increase risk for heavy smoking. Mol Psychiatry

Besson M, David V, Suarez S, Cormier A, Cazala P, Changeux JP, Granon S (2006) Genetic dissociation of two behaviors associated with nicotine addiction: β2-containing nicotinic receptors are involved in nicotine reinforcement but not in withdrawal syndrome. Psychopharmacology 187:189-199.

Biala G and Weglinska B (2005) Blockade of the expression of mecamylamine-precipitated nicotine withdrawal by calcium channel antagonists. Pharmacol Res 51:483-488.

Booker TK, Allen RS, Marks MJ, Grady SR, Whiteaker P, Smith KW, Collins AC, and Heinemann SF (1999) In: neuronal nicotinic receptors: from structure to therapeutics. Venice, p. 22

Breese CR, Marks MJ, Logel J, Adams CE, Sullivan B, Collins AC, Leonard S (1997) Effect of smoking history on [^{3}H]nicotine binding in human postmortem brain. J Pharmacol Exp Ther 282:7-13.

Brody AL, Mandelkern MA, London ED, et al (2006) Cigarette smoking saturates brain alpha 4 beta 2 nicotinic acetylcholine receptors. Arch Gen Psychiatry 161:1211-1218.

Bruijnzeel AW and Markou A (2004) Adaptations in cholinergic transmission associated with the affective signs of nicotine withdrawal. Neuropharmacology 47: 572-579.

Brunzell DH, Russell DS, Picciotto MR (2003) *In vivo* nicotine treatment regulates mesocorticolimbic CREB and ERK signaling in C57Bl/6J mice. J Neurochem 84:1431-1441.

Carboni E, Bontone L, Giua C, Di Chiara G (2000) Dissociation of physical abstinence signs from changes in extracelluar dopamine in the nucleus accumbens and in the prefrontal cortex of nicotine dependent rats. Drug Alcohol Depend 58:93-102.

Carpenter MJ, Upadhyaya HP, LaRowe SD, Saladin ME, Brady KT (2006) Menstrual cycle phase effects on nicotine withdrawal and cigarette craving: a review. Nicotine Tob Res 8:627-638.

Cartier GE, Yoshikami D, Gray WR, Luo S, Olivera BM, McIntosh JM (1996) A new α-conotoxin which targets α3β2 nicotinic acetylcholine receptors. J Biol Chem 271:7522-7528.

Champtiaux N, Han ZY, Bessis A, Rossi FM, Zoli M, Marubio L, McIntosh JM, Changeux JP (2002) Distribution and pharmacology of alpha 6-containing nicotinic acetylcholine receptors analyzed with mutant mice. J Neurosci 2:1208-1217.

Champtiaux N, Gotti C, Cordero-Erausquin M, David DJ, Przybylski C, Léna C, Clementi F, Moretti M, Rossi FM, Le Novère N, McIntosh JM, Gardier AM, Changeux JP (2003) Subunit composition of functional nicotinic receptors in dopaminergic neurons investigated with knock-out mice. J Neurosci 23:7820-7829.

Changeux JP, Bertrand D, Corringer PJ, Dehaene S, Edelstein S, Léna C, Le Novère N, Marubio L, Picciotto M, Soli M (1998) Brain nicotinic receptors:structure and regulation, role in learning and reinforcement. Brain Res 2-3:198-216. Review.

Cheeta S, Irvine EE, Kenny PJ, File SE (2001) The dorsal raphé nucleus is a crucial structure mediating nicotine's anxiolytic effects and the development of tolerance and withdrawal responses. Psychopharmacology 155:78-85.

Cryan JF, Bruijnzeel AW, Skjei KL, Markou A (2003) Bupropion enhances brain reward function and reverses the affective and somatic aspects of nicotine withdrawal in the rat. Psychopharmacology 168: 347-358.

Cummings KM and Mahoney M (2006) Current and emerging treatment approaches for tobacco dependence. Curr Oncol Rep 8:475-483.

Dajas-Bailador FA, Mogg AJ, Wonnacott S (2002) Intracellular Ca^{2+} signals evoled by stimulation of nicotinic acetylcholine receptors in SH-SY5Y cells: contribution of voltage-operated Ca^{2+} channels and Ca^{2+} stores. J Neurochem 81:606-614.

Dajas-Bailador FA and Wonnacott S (2004) Nicotinic acetylcholine receptors and the regulation of neuronal signaling. Trends Pharmacol Sci 25:317-324.

Damaj MI (2000) The involvement of spinal calcium/calmodulin-protein kinase II in nicotine-induced antinociception in mice. Eur J Pharmacol 404:103-110.

Damaj MI (2001) Influence of gender and sex hormones on nicotine acute and pharmacological effects in mice. J Pharmacol Exp Ther 296:132-140.

Damaj MI and Flores CM (2002). Nicotinic Receptors in the Nervous System. CRC Press; Boca Raton.

Damaj MI, Kao W, Martin BR (2003) Characterization of spontaneous and precipitated nicotine withdrawal in the mouse. J Pharmacol Exp Ther 307:526-534.

Damaj MI (2005) Calcium-acting drugs modulate expression and development of nicotine-induced antinociception in mice. J Pharmacol Exp Ther 315: 959-964.

Dani JA and Heinemann S (1996) Molecular and cellular aspects of nicotine abuse. Neuron 16:905-908.

Dani JA, Ji D, Zhou FM (2001) Synaptic plasticity and nicotine addiction. Neuron 31:349-352.

Davis JA, James JR, Siegel SJ, Gould TJ (2005) Withdrawal from chronic nicotine administration impairs contextual fear conditioning in C57Bl/6 mice. J. Neurosci 25: 8708-8713.

De Biasi M (2002) Nicotinic receptor mutant mice in the study of autonomic function. Curr Drug Targets CNS Neurol Disord 4:331-336.

De Biasi M and Salas R (2008) Influence of neuronal nicotinic receptors over nicotine addiction and withdrawal. Exp Biol Med (Maywood) 233:917-929.

Debruyne D, Sobrio F, Hinschberger A, Camsonne R, Coquerel A, Barré L (2003) Short-term pharmacokinetics and brain distribution of mecamylamine as a preliminary to carbon-11 labeling for nicotinic receptor investigation. J Pharm Sci 92:1051-1057.

De Camilli P, Benfenati F, Valtorta F, Greengard P (1990) The synapsins. Annu Rev Cell Biol 6:433-460.

Deisseroth K, Heist EK, Tsien R (1998) Translocation of calmodulin to the nucleus supports CREB phosphorylation in hippocampal neurons. Nature 392:198-202.

Dizgah IM, Karimian SM, Zarrindast MR, Sohanaki H (2005) Attenuation of morphine withdrawal signs by a D1 receptor agonist in the locus coeruleus of rats. Neuroreport 16:1683-1686.

Enslen H, Sun P, Brickey D, Soderling SH, Klamo E, Soderling TR (1994) Characterization of Ca2+/calmodulin-dependent kinase IV. Role in transcriptional regulation. J Biol Chem 269:155520-1555207.

Esmaeili-Mahani S, Fathi Y, Motamedi F, Hosseinpanah F, ahmadiani A (2008) L-type calcium channel blockade attenuates morphine withdrawal: in vivo interaction between L-type calcium channels and corticosterone. Horm Behav 57: 351-357.

Fan G, Wang L, Qiu H, Ma L, Pei G (1999) Inhibition of calcium/calmodulin-dependent protein kinase II in rat hippocampus attenuates morphine tolerance and dependence. Mol Pharmacol 56:39-45.

Flora A, Schulz R, Benfante R, Battaglioli E, Terzano S, Clementi F, Fornasari D (2002) Neuronal and extraneuronal expression and regulation of the human alpha 5 nicotinic receptor subunit gene. J Neurochem 75:18-27.

Foreman NP (1983) Head-dipping in rats with superior collicular, medial frontal cortical, and hippocampal lesions. Physiol Behav 30:711-717.

Fung YK, Schmid MJ, Anderson TM, Lau YS (1996) Effects of nicotine withdrawal on central dopaminergic systems. Pharmacol Biochem Behav 53:635-640.

Gao L, Blair L, Marshall J (2006) CaMKII-dependent effects of KN93 and its inactive analog KN92: reversible inhibition of L-type calcium channels. Biochem Biophys Res Commun 345:1606-1610.

Gerzanich V, Wang F, Kuryatov A, Lindstrom J (1998) Alpha 5 subunit alters desensitization, pharmacology, Ca++ permeability, and Ca++ modulation of human neuronal alpha 3 nicotinic receptors. J Pharmacol Exp Ther 286: 311-320.

Grabus SD, Martin BR, Damaj MI (2005) Nicotine physical dependence in the mouse: involvement of the α7 nicotinic receptor subtype. Eur J Pharmacol 515:90-93.

Greengard P, Valtorta F, Czernik AJ, Benfenati F (1993) Synaptic vesicle phosphoproteins and regulation of synaptic function. Science 259:780-785.

Grenhoff J, Aston-Jones G, Svensson TH (1986) Nicotinic effects on the firing pattern of midbrain dopamine neurons. Acta Physiol Scand 128:351-358

Gonzales D, Rennard SI, Nides M, Oncken C, Azoulay S, Billing CB et al. (2006) Veranicline, and α4β2 nicotinic acetylcholine receptor partial agonist vs. sustained-release buproprion and placebo for smoking cessation. J Am Med Assoc 296:47-55.

Glick SD, Maisonneuve JM, Dickinson HA (2000) 18-MC reduces methamphetamine and nicotine self-administration in rats. Neuroreport 11:2013-2015.

Glick SD, Maisonneuve IM, Kitchen BA, Fleck MW (2002) Antagonism of α3β4 nicotinic receptors as a strategy to reduce opioid and stimulant self-administration. Eur J Pharmacol 438:99–105.

Glick SD, Ramirez RL, Livi JM, Maisonneuve IM (2006) 18-Methoxycoronaridine acts in the medial habenula and/or interpeduncular nucleus to decrease morphine self-administration in rats. Eur J Pharmacol 537:94-98.

Guillem K, Vouillac C, Koob GF, Cador M, Stinus L (2007) Monoamine oxidase inhibition dramatically prolongs the duration of nicotine withdrawal-induced place aversion. Biol Psychiatry 63:158-163.

Harrison AA, Liem YTB, Markou A (2001) Fluoxetine combined with a serotonin-1A receptor antagonist reversed reward deficits observed during nicotine and amphetamine withdrawal in rats. Neuropsychopharmacology 25:55-71.

Hicks A, Davis S, Rodger J, Helme-Guizon A, Laroche S, Mallet J (1997) Synapsin I and syntaxin 1B: key elements in the comtrol of neurotransmitter release are regulated by neuronal activation and long-term potentiation in vivo. Neuroscience 79:329-340.

Hildebrand BE, Nomikos GG, Bondjers C, Nisell M, and Svensson TH (1997) Behavioral manifestations of the nicotine abstinence syndrome in the rat: peripheral versus central mechanisms. Psychopharmacology (Berl) 129:348-356.

Hildebrand BE, Nomikos GG, Hertel P, Schilström B, Svensson TH (1998) Reduced dopamine output in the nucleus accumbens but not in the medial prefrontal cortex in rats displaying a mecamylamine-precipitated nicotine withdrawal syndrome. Brain Res 779:214-225

Hildebrand BE, Panagis G, Svensson TH, Nomikos GG (1999) Behavioral and biochemical manifestations of mecamylamine-precipitated nicotine withdrawal in the rat: role of nicotinic receptors in the ventral tegmental area. Neuropsychopharmacology 21:559-574.

Hilfiker S, Pieribone VA, Czernik AJ, Kao HT, Augustine GJ, and Greengard P (1999) Synapsins as regulators of neurotransmitter release. Philos Trans R Soc Lond B Biol Sci 354:269-279.

Hilleman DE, Mohiuddin SM, Del Corc MG, Sketch MH (1992) Effect of buspirone on withdrawal symptoms associated with smoking cessation. Arch Intern Med 52:350-352.

Hook SS and Means AR (2001) Ca2+/CaM-dependent kinases: from activation to function. Annu Rev Pharmacol Toxicol 41:471-505. Review.

Hughes JR, Gulliver SB, Fenwick JW et al. (1992) Smoking cessation among self-quitters. Health Psychol 11:331-334.

Hughes JR (2006) Clinical significance of tobacco withdrawal. Nicotine Tob Res 8:153-156.

Hughes JR (2007) Effects of abstinence from tobacco: valid symptoms and time course. Nicotine Tob Res 9:315-327.

Hyman SE (2005) Addiction: a disease of learning and memory. Am J Psychiatry 162:1414-1422.

Hyman SE, Malenka RC, Nestler EJ (2006) Neural mechanisms of addiction: the role of reward-related learning and memory. Annu Rev Neurosci 29:565-598. Review.

Irvine EE, Cheeta S, File SE (2001) Tolerance to nicotine's effects in the elevated plus-maze and increased anxiety during withdrawal. Pharmacol Biochem Behav 68:319-325.

Ise Y, Narita M, Nagase H, Suzuki T, (2002) Modulation of κ-opiodergic systems on mecamylamine-precipitated nicotine-withdrawal aversion in rats. Neurosci Lett 323: 164-166.

Iwata S, Hewlett GH, Ferrell ST, Czernik AJ, Meiri KF, Gnegy ME (1996) Increases *in vivo* phosphorylation state of neuromodulin and synapsin I in striatum from rats treated with repeated amphetamine. J Pharmacol Exp Ther 278:1428-1434.

Iwata S, Hewlett GH, Ferrell ST, Kantor L, Gnegy ME (1997) Enhanced dopamine release and phosphorylation of synapsin I and neuromodulin in striatal synaptosomes after repeated amphetamine. J Pharmacol Exp Ther 283:1445-1452.

Iwata S, Hewlett GH, Gnegt ME (1997) Amphetamine increases the phosphorylation of neuromodulin and synapsin I in rat striatal synaptosomes. Synapse 26:281-291.

Jackson KJ, Martin BR, Changeux JP, Damaj MI (2008) Differential role of nicotinic acetylcholine receptor subunits in physical and affective nicotine withdrawal signs. J Pharmacol Exp Ther 325: 302-312.

John U, Meyer C, Rumpf HJ, Hapke U (2008) Nicotine dependence criteria and nicotine withdrawal symptoms in relation to pain among an adult general population sample. Eur J Pain (Epub ahead of print).

Johnson PM, Hollander JA, Kenny PJ (2008) Decreased brain reward function during nicotine withdrawal in C57Bl6 mice; evidence from intracranial self stimulation (ICSS) studies. Pharmacol Biochem Behav 90: 409-415.

Jorenby DE, Hays JT, Rigotti NA, Azoulay NA, Watsky EJ, Williams KE, Billing CB, Gong J, Reeves KR (2006) Efficacy of varenicline, an α4β2 nicotinic acetylcholine

receptor partial agonist, vs placebo or sustained-release bupropion for smoking cessation. J Am Med Assoc 296:56-63.

Kano T, Suzuki Y, Shibuya M, Kiuchi K, Hagiwara M (1995) Cocaine-induced CREB phosphorylation and cFos expression are suppressed in Parkinsonism model mice. NeuroReport 6:2197-2200.

Karler R, Finnegan KT, Calder LD (1993) Blockade of behavioral sensitization to cocaine and amphetamine by inhibitors of protein synthesis. Brain Res 603:19-24.

Katsura M, Mohri Y, Shuto K, Hai-Du Y, Amano T, Tsujimura A, Sasa M, Ohkuma S (2002) Up-regulation of L-type voltage-dependent calcium channels after long term exposure to nicotine in cerebral cortical neurons. J Biol Chem 277:7979-7988.

Katz B and Thesleff S (1957) A study of the desensitization produced by acetylcholine at the motor end plate. J Physiol 138:63-80.

Kenny PJ and Markou A (2001) Neurobiology of the nicotine withdrawal syndrome. Pharmacol Biochem Behav 70:531-549.

King SL, Caldarone BJ, Picciotto MR (2004) Beta2-subunit-containing nicotinic acetylcholine receptors are critical for dopamine-dependent locomotor activation following repeated nicotine administration. Neuropharmacology 47:132-139.

Klink R, de Kerchove d'Exaerde A, Zoli M, Changeux JP (2001) Molecular and physiological diversity of nicotinic acetylcholine receptors in the midbrain dopaminergic nuclei. J Neurosci 5:1452-1463.

Koob GP, Heinrichs SC, Pich EM, Menzaghi F, Baldwin H, Miczek K, Britton KT (1993) The role of corticotropin-releasing factor in behavioral responses to stress. Ciba Found Symp 172:277-289.

Kota D, Martin BR, Robinson SE, Damaj MI (2007) Nicotine dependence and reward differ between adolescent and adult male mice. J Pharmacol Exp Ther 322:399-407.

Kota D, Martin BR, Damaj MI (2008) Age-dependent differences in nicotine reward and withdrawal in female mice. Psychopharmacology 198:201-210.

Kuryatov A, Olale F, Cooper J, Choi C, Lindstrom J (2000) Human α6 AChR subtypes: subunit composition, assembly and pharmacology responses. Neuropharmacology 39:2570-2590.

Lai A, Parameswaran N, Khwaja M, Whiteaker P, Lindstrom JM, Fan H, McIntosh JM, Grady SR, Quik M (2005) Long-term nicotine treatment decreases striatal α6* nicotinic acetylcholine receptor sites and function in mice. Mol Pharmacol 67:1639-1647.

Lane-Ladd SB, Pineda J, Boundy VA, Pfeuffer T, Krupinski J, Aghajanian GK, Nestler EJ (1997) CREB (cAMP response element-binding protein) in the locus coeruleus: biochemical, physiological, and behavioral evidence for a role in opiate dependence. J Neurosci 17:7890-7901.

Laviolette SR, Alexson TO, van der Kooy D (2002) Lesions of the tegmental pedunculopontine nucleus block the rewarding effects and reveal the aversive effects of nicotine in the ventral tegmental area. J Neurosci 22:8653-8660.

Laviolette SR, Lauzon NM, Bishop SF, Sun N, Tan H (2008) Dopamine signaling through D1-like versus D2-like receptors in the nucleus accumbens core versus shell differentially modulates nicotine reward sensitivity. J Neurosci 28:8025-8033.

Le Novère N and Changeux JP (1995) Molecular evolution of the nicotinic acetylcholine receptor: an example of multigene family in excitable cells. J Mol Evol 40:155-172.

Le Novère N, Zoli M, Changeux JP (1996) Neuronal nicotinic receptor alpha 6 subunit mRNA is selectively concentrated in catecholaminergic nuclei of the rat brain. Eur J Neurosci 8:2428-2349.

Le Novère N, Zoli M, Lena C, Ferrari R, Picciotto MR, Merlo-Pich E, Changeux JP (1999) Involvement of alpha6 nicotinic receptor subunit in nicotine-elicited locomotion,demonstrated by in vivo antisense oligonucleotide infusion. Neuroreport 10:2497-2501.

Liang D, Li X, Clark JD (2004) Increased expression of Ca2+/calmodulin-dependent protein kinase IIα during chronic morphine exposure. Neuroscience 13:769-775.

Licata SC, Schmidt HD, Pierce RC (2004) Suppressing calcium/calmodulin-dependent protein kinase II in the ventral tegmental area enhances the acute behavioural response to cocaine but attenuates the initiation of cocaine-induced behavioural sensitization in rats. Eur J Neurosci 19:405-414.

Liu JJ, Mohila CA, Gong Y, Govindarajan N, Onn SP (2005) Chronic nicotine exposure during adolescence differentially influences calcium-binding in rat anterior cingulate cortex. Eur J Neurosci 22:2462-2474.

Liu Q and Berg DK (1999) Actin filaments and the opposing actions on CaM kinase II and calcineurin in regulating α7-containing nicotinic receptors on chick ciliary ganglion neurons. J Neurosci 19:10280-10288.

Liu ZH and Jin WQ (2004) Decrease of ventral tegmental area dopamine neuronal activity in nicotine withdrawal rats. Neuroreport 15:1479-1481.

Lisman J, Schulman H, Cline H (2002) The molecular basis of CaMKII function in synaptic and behavioral memory. Nature 3:175-90.

Llinás R, Gruner JA, Sugimori M, McGuinness TL, Greengard P (1991) Regulation of synapsin I and Ca2+-calmodulin dependent protein kinase II of transmitter release in squid giant synapse. J Physiol 436:257-282.

Luo S, Kulak JM, Cartier GE, Jacobsen RB, Yoshikami D, Olivera BM, McIntosh JM (1998) α-Conotoxin AuIB selectively blocks α3β4 nicotinic acetylcholine receptors and nicotine-evoked norepinephrine release. J Neurosci 18:8571-8579.

Lynch MA, Voss KL, Rodriguez J, Bliss TV (1994) Increase in synaptic vesicle proteins accompanies long-term potentiation in the dentate gyrus. Neuroscience 60:1-5.

MacNicol M and Schulman H (1992) Multiple Ca^{2+} signaling pathways converge on CaM kinase in PC12 cells. FEBS Lett 304:237-240.

Malenka RC, Kauer JA, Perkel DJ, Mauk MD, Kelly PT, Nicoll RA, Waxham MN (1989) An essential role for postsynaptic calmodulin and protein kinase activity in long-term potentiation. Nature 340:554-557

Malenka RC and Bear MF (2004) LTP and LTD: an embarrassment of riches. Neuron 44:5-21.

Malin DH, Lake JR, Newlin-Maultsby P, Roberts LK, Lanier JG, Carter VA, Cunningham JS, Wilson OB (1992) Rodent model of nicotine abstinence syndrome. Pharmacol Biochem Behav 43:779-784.

Malin DH, Lake JR, Smith TD, Khambati HN, Meyers-Paal RL, Montellano AL, Jennings RE, Erwin DS, Presley SE, Perales BA (2006) Bupropion attenuates nicotine abstinence syndrome in the rat. Psychopharmacology 184:494-503.

Manji HK, Drevets WC, Charney DS (2001) The cellular neurobiology of depression. Nature Med 7:541-547.

Mansvelder HD and McGehee DS (2002) Cellular and synaptic mechanisms of nicotine addiction. J Neurobiol 53:606-617. Review.

Martin SJ, Grimwood PD, Morris RG (2000) Synaptic plasticity and memory: an evaluation of the hypothesis. Annu Rev Neurosci 23:649-711. Review.

Martin, T (2002). Global smoking statistics for 2002: overall stats and youth smoking facts. Retrieved June 2008 from

http://quitsmoking.about.com/cs/antismoking/a/statistics.htm

Markou A, Kosten TR, Koob GF (1998) Neurobiological similarities in depression and drug dependence: A self-medication hypothesis. Neuropsychopharmacology 18:135-174.

Marubio LM, del Mar Arroyo-Jimenez M, Cordero-Erausquin M, Lena C, Le Novere N, de Kerchove d'Exaerde A, Huchet M, Damaj MI, Changeux JP (1999) Reduced antinociception in mice lacking neuronal nicotinic receptor subunits. Nature 398:805-810.

Matta SG, McCoy JG, Foster CA, Sharp BM (1995) Nicotinic agonists administered into the fourth ventricle stimulate norepinephrine secretion on the hypothalamic paraventricular nucleus: an in vivo microdialysis study. Neuroendocrinology 61:383-392.

Matthews RP, Guthrie CR, Wailes LM, Zhao X, Means AR, McKnight GS (1994) Calcium/calmodulin-dependent protein kinase types II and IV differentially regulate CREB-dependent gene expression. Mol Cell Biol 14:6107-6116.

Matus-Leibovitch N, Ezra-Macabee VE, Saya D, Attali B, Avidor-Reiss T, Barg J, Vogel Z (1995) Increased expression of synapsin I mRNA in defined areas of the rat central nervous system following chronic morphine treatment. Mol Brain Res 34:221-230.

McGehee DS and Role LW (1995) Physiological diversity of nicotinic acetylcholine receptors expressed by vertebrate neurons. Annu Rev Physiol 57:521-546.

McIntosh JM, Azam L, Staheli S, Dowell C, Lindstrom JM, Kuryatov A, Garrett JE, Marks MJ, Whiteaker P (2004) Analogs of α-conotoxin MII are selective for α6-containing nicotinic acetylcholine receptors. Mol Pharmacol 65:944-952.

Mendrek A, Monterosso J, Simon SL, Jarvik M, Brody A, Olmstead R, Domier CP, Cohen MS, Ernst M, London ED (2006) Working memory in cigarette smokers: Comparison to non-smokers and effects in abstinence. Addict Behav 31:833-844.

Michaluk J, Karolewicz B, Antiewicz-Michaluk L, Vetulani J (1998) Effects of various Ca^{2+} channel antagonists on morphine analgesia, tolerance, and dependence, and on blood pressure in the rat. Eur J Pharmacol 352:189-197.

Mogg AJ, Whiteaker P, McIntosh JM, Marks M, Collins AC, Wonnacott S (2002) Methyllycaconitine is a potent antagonist of alpha-conotoxin-MII-sensitive presynaptic nicotinic acetylcholine receptors in rat striatum. J Pharmacol Exp Ther 302:197-204.

Morris JS, Smith KA, Cowen PJ, Friston KJ, Dolan RJ (1999) Covariation of activity in habenula and dorsal raphé nuclei following tryptophan depletion. Neuroimage 10:163-172.

Moser N, Mechawar N, Jones I, Gochberg-Sarver A, Orr-Urtreger A, Plomann M, Salas R et al. (2007) Evaluating the suitability of nicotinic acetylcholine receptor antibodies for standard immunodetection procedures. J Neurochem 102:479-492.

Miura M, Ishii K, Aosaki T, Sumikawa K (2006) Chronic nicotine treatment increases GABAergic input to striatal neurons. Neuroreport 17:537-540.

Miyabe T, Miletic V (2005) Multiple kinase pathways mediate the early sciatic ligation-associated activation of CREB in the rat spinal dorsal horn. Neurosci Lett 381:80-85.

Nadeau JH (2001) Modifier genes in mice and humans. Nat Rev Genet 2:165-174.

Narita M, Matsumura Y, Ozaki S, Ise Y, Yajima Y, Suzuki T (2004) Role of the calcium/calmodulin-dependent protein kinase II (CaMKII) in the morphine-induced pharmacological effects in the mouse. Neuroscience 126:415-421.

Naylor C, Quarta D, Fernandes C, Stolerman IP (2005) Tolerance to nicotine in mice lacking alpha7 nicotinic receptors. Psychopharmacology (Berl) 180:558-563.

Nestler EJ and Ahhajanian GK (1997) Molecular and cellular basis of addiction. Science 278:58-63.

Nestler EJ and Carlezon WA (2006) The mesolimbic dopamine reward circuit in depression. Biol Psychiatry 59:1151-1159.

Nestler EJ, Hyman SE, Malenka RC (2001a) Molecular basis of neuropharmacology. McGraw-Hill: New York.

Nestler EJ (2001b) Molecular basis of long-term plasticity underlying addiction. Nature Rev Neurosci 2:119-128.

Nestler EJ (2002) Common molecular and cellular substrates of addiction and memory. Neurobiol Learn Mem 78:637-647.

Nichols RA, Sihra TS, Czernik AJ, Mairn AC, Greengard P (1990) Calcium/calmodulin-dependent protein kinase II increases glutamate and noradrenaline release from synaptosomes. Nature 343:647-651.

Nomikos GG, Hildebrand BE, Panagis G, Svensson TH (1999) Nicotine withdrawal in the rat: role of α7 nicotinic receptors in the ventral tegmental area. Neuropharmacology 10: 697-702.

O'Dell LE, Torres OV, Natividad LA, Tejeda HA (2007) Adolescent nicotine exposure produces less affective measures of withdrawal relative to adult nicotine exposure in male rats. Neurotoxicol Teratol 29:17-22.

Orr-Urtreger A, Göldner FM, Saeki M, Lorenzo I, Goldberg L, De Biasi M, Dani JA, Patrick JW, Beaudet AL (1997) Mice deficient in the alpha 7 neuronal nicotinic acetylcholine receptor lack α-bungarotoxin binding sites and hippocampal fast nicotinic current. J Neurosci 17:9165-9171

Panchal V, Taraschenko OD, Maisonneuve IM, Slick GD (2005) Attenuation of morphine withdrawal signs by intracerebral administration of 18-methoxycoronaridine. Eur J Pharmacol 525:98-104.

Pandey SC, Roy J, Xu T, Mittal N (2001) Effects of protracted nicotine exposure and withdrawal on the expression and phosphorylation of the CREB gene transcription factor in rat brain. J Neurochem 77:943-952.

Papke RL, Sanberg PR, Shytle RD (2001) Analysis of mecamylamine stereoisomers on human nicotinic subtypes. J Pharmacol Exp Thera 297:646-656.

Papke RL and Thinschmidt JS (1998) The correction of alpha7 nicotinic acetylcholine receptor concentration-response relationships in Xenopus oocytes. Neurosci Lett 256:163-166.

Picciotto MR, Zoli M, Léna C, Bessis A, Lallemand Y, LeNovère N, Vincent P, Pich EM, Brûlet P, Changeux JP (1995) Abnormal avoidance learning in mice lacking functional high-affinity nicotine receptor in the brain. Nature 374:65-67.

Picciotto MR, Zoli M, Rimondini R, Lèna C, Marubio LM, Pich EM, Fuxe K, Changeux JP (1998) Acetylcholine receptors containing the β2 subunit are involved in the reinforcing properties of nicotine. Nature 391:173-177.

Pidoplichko VI, De Biasai M, Williams JT, Dani JA (1997) Nicotine activates and desensitizes midbrain dopamine neurons. Nature 390:401-404.

Pidoplichko VI, Noguchi J, Areola OO, Liang Y, Peterson J, Zhang T, Dani JA (2004) Nicotinic cholinergic synaptic mechanisms in the ventral tegmental area contribute to nicotine addiction. Learn Mem 11:60-69.

Pierce RC and Kalivas PW (1997) Repeated cocaine modifies the mechanism by which amphetamine releases dopamine. J Neurosci 17:3254-3261.

Pierce RC, Quick EA, Reeder DC, Morgan ZR, Kalivas PW (1998) Calcium-mediated second messengers modulate the expression of behavioral sensitization to cocaine. J Pharmacol Exp Ther 286:1171-1176.

Pluzarev O and Pandey SC (2004) Modulation of CREB expression and phosphorylation in the rat nucleus accumbens during nicotine exposure and withdrawal. J Neurosci Res 77:884-891.

Pontieri FE, Tanda G, Orzi F, Di Chiara G (1996) Effects of nicotine on the nucleus accumbens and similarity to those of addictive drugs. Nature 382:255-257

Portugal GS, Kenney JW, Gould TJ (2008) Beta2 subunit containing acetylcholine receptors mediate nicotine withdrawal deficits in the acquisition of contextual fear conditioning. Neurobiol Learn Mem 89:106-113.

Quick MW, Ceballos RM, Kasten M, McIntosh JM, Lester RA (1999) α3β4-subunit containing nicotinic receptors dominate function in rat medial habenula neurons. Neuropharmacology 38:769-783.

Rada P, Jensen K, Hoebel BG (2001) Effects of nicotine and mecamylamine-induced withdrawal in extracellular dopamine and acetylcholine in the rat nucleus accumbens. Psychopharmacology 157:105-110

Rathouz MM and Berg DK (1994) Synaptic-type acetylcholine receptors raise intracellular calcium levels in neurons by two mechanisms. J Neurosci 14:6935-6945.

Robbins TW and Everitt BJ (2002) Limbic-striatal memory systems and drug addiction. Neurobiol Learn Mem 78:625-636.

Role LW and Berg DK (1996) Nicotinic receptors in the development and modulation of CNS synapses. Neuron 16:1077-1085.

Ross SA, Wong JY, Clifford JJ, Kinsella A, Massalas JS, Horne MK, Scheffer IE, Kola I, Waddington JL, Berkovic SF, Drago J (2000) Phenotypic characterization of an

alpha 4 neuronal nicotinic acetylcholine receptor subunit knock-out mouse. J Neurosci 20:6431-6441.

Salas R, Orr-Urtreger A, Broide RS, Beaudet A, Paylor R, De Biasi M (2003) The nicotinic acetylcholine receptor subunit alpha 5 mediates short-term effects of nicotine in vivo. Mol Pharmacol 63:1059-1066.

Salas R, Pieri F, De Biasi M (2004) Decreased signs of nicotine withdrawal in mice null for the β4 nicotinic acetylcholine receptor subunit. J Neurosci 24:10035-10039.

Salas R, Main A, Gangitano D, De Biasi M (2007) Decreased withdrawal symptoms but normal tolerance in mice null for the alpha7 nicotinic acetylcholine receptor subunit. Neuropharmacology 53:863-869.

Salminen O, Murphy KL, McIntosh JM, Drago J, Marks MJ, Collins AC, Grady SR (2004) Subunit composition and pharmacology of two classes of striatal presynaptic nicotinic acetylcholine receptors mediating dopamine release in mice. Mol Pharmacol 65:1526-1535.

Schmidt BL, Tambeli CH, Gear RW, Levine JD (2001) Nicotine withdrawal hyperalgesia and opioid-mediated analgesia depend on nicotine receptors in nucleus accumbens. Neuroscience 106:129-136.

Schneider M, Spanagel R, Zhang S, Bading H, Klugmann M (2007) Adeno-associated virus (AAV)-mediated suppression of Ca2+/calmodulin kinase IV activity in the nucleus accumbens modulates emotional behavior in mice. BMC Neurosci 8:105-113.

Schneider NG, Olmstead RE, Steinberg C, Sloan K, Daims RM, Brown HV (1996) Efficacy of buspirone in smoking cessation:a placebo-controlled trial. Clin Pharmacol Ther 60:568-575.

Schulman H and Hanson PI (1993) Multifunctional Ca^{2+} /calmodulin-dependent protein kinase. Neurochem Res 18:65-77.

Shaw-Lutchman TZ, Impey S, Storm D, Nestler EJ (2003) Regulation of CRE-mediated transcription in mouse brain by amphetamine. Synapse 48:10-17.

Sheffield EB, Quick MW, Lester RA (2000) Nicotinic acetylcholine receptor subunit mRNA expression and channel function in medial habenula neurons. Neuropharmacology 39:2591-2603.

Shoaib M, Gommans J, Morley A, Stolerman IP, Grailhe R, Changeux JP (2002) The role of nicotinic receptor beta-2 subunits in nicotine discrimination and conditioned taste aversion. Neuropharmacology 45:530-539.

Silva AJ, Stevens CF, Tonegawa S, Wang Y (1992) Deficient hippocampal long-term potentiation in α-calcium calmodulin kinase II mutant mice. Science 257:201-205.

Silva AJ, Paylor R, Wehner JM, Tonegawa S (1992) Impaired spatial learning in α-calcium-calmodulin kinase II mutant mice. Science 257:206-211.

Slemmer JE, Martin BR, Damaj MI (2000) Bupropion is a nicotinic antagonist. J Pharmacol Exp Ther 295:321-327.

Steiner RC, Heath CJ, Picciotto MR (2007) Nicotine-induced phosphorylation of ERK in mouse primary cortical neurons: evidence for involvement of glutamatergic signaling and CaMKII. J Neurochem 103: 666- 678.

Stoker AK, Semenova S, Markou A (2008) Affective and somatic aspects of spontaneous and precipitated nicotine withdrawal in C57Bl/6J and BALB/cByJ mice. Neuropharmacology 54:1223-1232.

Stolerman IP and Shoaib M (1991) The neurobiology of tobacco addiction. Trends Pharmacol Sci 12: 467-473.

Stolerman IP and Jarvis MJ (1995) The scientific case that nicotine is addictive. Psychopharmacology 117:2-10.

Sun P, Enslen H, Myung PS, Maurer RA (1994) Differential activation of CREB by Ca2+/calmodulin-dependent protein kinases type II and type IV involves phosphorylation of a site that negatively regulates activity. Gene Dev 8:2527-2539.

Suzuki T, Ise Y, Tsuda M, Maeda J, Misawa M (1996) Mecamylamine-precipitated nicotine-withdrawal aversion in rats. Eur. J. Pharmacol 314: 281-284.

Suzuki T, Ise Y, Maeda J, Misawa M (1999) Mecamylamine-precipitated nicotine-withdrawal aversion in lewis and fischer 344 inbred rat strains. Eur J Pharmacol 369:159-162.

Swan GE, Ward MM, Jack LM (1996) Abstinence effects as predictors of 28-day relapse in smokers. Addict Behav 21:481-490.

Tan SE (2002) Impairing the amphetamine conditioning in rats through the inhibition of hippocampal calcium/calmodulin-dependent protein kinase II activity. Neuropharmacology 42:540-547.

Tang L, Shukla PK, Wang LX, Wang ZJ (2006) Reversal of morphine antinociceptive tolerance and dependence bythe acute supraspinal inhibition of Ca2+/calmodulin-dependent protein kinase II. J Pharmacol Exp Ther 317:901-909.

Taraschenko OD, Shulan JM, Maisonneuve IM, Glick SD (2007) 18-MC acts in the medial habenula and interpeduncular nucleus to attenuate dopamine sensitization to morphine in the nucleus accumbens. Synapse 61:547-560.

Teitelbaum H and Milner P (1963) Activity changes following partial hippocampal lesions in rats. J Comp Physiol Psychol 56:284-289.

Tiraboschi E, Giambelli R, D'Urso G, Galietta A, Barbon A, de Bartolomeis A, Gennarelli M, Barlati S, Racagni G, Popoli M (2004) Antidepressants activate CaMKII in neuron cell body by Thr286 phosphorylation. Neuroreport 15:2393-2396.

US Department of Health and Human Sciences (1988) The health consequences of smoking: nicotine addiction. A report of the Surgeon General Maryland: Office on Smoking and Health.

Vetter DE, Liberman MC, Mann J, Barhanin J, Boulter J, Brown MC, Saffiote-Kolman J, Heinemann SF, Elgoyhen AB (1999) Role of alpha9 nicotinic Ach receptor subunits in the development and function of cochlear efferent innervations. Neuron 23:93-103.

Vitcheva V and Mitcheva M (2004) Effects of nifedipine on behavioral and biochemical parameters in rats after multiple morphine administration. Methods Find Exp Clin Pharmacol 26:631-634.

Wada E, Wada K, Boulter J, Deneris E, Heinemann W, Patrick J, and Swanson LW (1989) Distribution of α2, α3, α4, and β2 neuronal nicotinic acetylcholine receptor subunit mRNAs in the central nervous system: A hybridization histochemical study in the rat. J Comp Neurol 289:314-335.

Wada E, McKInnon D, Heinemann S, Patrick J, Swanson LW (1990) The distribution of mRNA encoded by a new member of the neuronal nicotinic acetylcholine receptor gene family (alpha 5) in the rat central nervous system. Brain Res 1:45-53.

Walters CL, Blendy JA (2001) Different requirements for cAMP response element binding protein in positive and negative reinforcing properties of drugs of abuse. J Neurosci 21:9438-9444.

Walters CL, Cleck NJ, Kuo Y, Blendy JA (2005) μ-Opiod receptor and CREB activation are required for nicotine reward. Neuron 46:933-943.

Walters CL, Brown S, Changeux JP, Martin BR, Damaj MI (2006) The β2 but not α7 subunit of the nicotinic acetylcholine receptor is required for nicotine-conditioned place preference in mice. Psychopharmacology 184:339-344.

Walters CL and Damaj MI (2008) Calcium-calmodulin protein kinases mediate CREB phosphorylation in the mesolimbic dopamine pathway after acute nicotine treatment through β2-containing receptors. Submitted

Wang F, Gerzanich V, Wells GB, Anand R, Peng X, Keyser K, Lindstrom J (1996) Assembly of human neuronal nicotinic receptor alpha5 subunits with alpha3, beta2, and beta4 subunits. J Biol Chem 271:17656-17665.

Wang H, Shimizu E, Tang Y, Cho M, Kyin, Zuo W, Robinson DA et al. (2003) Inducible protein knockout reveals temporal requirement of CaMKII reactivation for memory consolidation in the brain. Proc Natl Acad Sci USA 100:4287-4292.

Wang ZJ, Tang L, Xin L (2003) Reversal of morphine antinociceptive tolerance by acute spinal inhibition of Ca^{2+}/calmodulin-dependent protein kinase II. Eur J Pharmacol 465:199-200.

Ward J, Cockcroft V, Lunt G, Smillie F, Wonnacott S (1990) Methyllycaconitine: a selective probe for neuronal α-bungarotoxin binding sites. FEBS Lett 270:45-48.

Ward MM, Swan GE, Jack LM (2001) Self-reported abstinence effects in the first month after smoking cessation. Addict Behav 26:311-327.

Watkins SS, Stinus L, Koob GF, Markou A (2000) Reward and somatic changes during precipitated nicotine withdrawal in rats: centrally and peripherally mediated effects. J Pharmacol Exp Ther 292:1053-1064.

West RJ, Hajek P, Belcher M (1989) Severity of withdrawal symptoms as a predictor of outcome of an attempt to quit smoking. Psychol Med 19:981-985.

Wheeler DG, Barrett CF, Tsien RW (2006) L-type calcium channel ligands block nicotine-induced signaling to CREB by inhibiting nicotinic receptors. Neuropharmacology 51:27-36.

Whiteaker P, Peterson CG, Xu W, McIntosh JM, Paylor R, Beaudet AL (2002) Involvement of the alpha3 subunit in central nicotinic receptor populations. J Neurosci 22: 2522-2529.

Wilkinson JL and Bevins RA (2008) Intravenous nicotine conditions a place preference in rats using an unbiased design. Pharmacol Biochem Behav 88:256-264.

Wonnacott S (1997) Presynaptic nicotinic ACh receptors. Trends Neurosci 20:92-98.

Wu X and McMurray CT (2001) Calmodulin kinase II attenuation of gene transcription by preventing cAMP response element-binding protein (CREB) dimerization and binding of the CREB-binding protein. J Biol Chem 276:1735-1741.

Xing G, Russell S, Hough C, O'Grady J. Zhang L, Yang S et al. (2002) Decreased prefrontal CaMKIIα mRNA in bipolar illness. Neuroreport 13:501-505.

Xu W, Gelber S, Orr-Urtreger A, Armstrong D, Lewis RA, Ou CN, Patrick J, Role L, De Biasi M, Beaudet AL (1999) Megacystis, mydriasis, and ion channel defect in mice lacking the alpha3 neuronal nicotinic acetylcholine receptor. Proc Natl Acad Sci USA 96:5746-5751.

Xu W, Orr-Uretreger A, Nigro F, Gelber S, Sutcliffe CB, Armstrong D, Patrick JW, Role LW, Beaudet AL, De Biasi M (1999) Multiorgan autonomic dysfunction in mice lacking the beta2 and the beta4 subunits of neuronal nicotinic acetylcholine receptors. J Neurosci 19:9298-9305.

Zanoveli JM, Ferreira-Netto C, Brandão ML (2007) Conditioned place aversion organized in the dorsal periaqueductal gray recruits the laterodorsal nucleus of the thalamus and the basolateral amygdala. Exp Neurol 208:127-136.

Zharkovsky A, Totterman AM, Moisio J, Ahtee L (1993) Concurrent nimodipine attenuates the withdrawal signs and the increase of cerebral dihydropyridine binding after chronic morphine treatment in rats. Naunyn-Schmiedeberg's Arch Pharmacol 347:483-486.

Zoli M, Le Novère N, Hill JA, Changeux JP (1995) Developmental regulation of nicotinic Ach receptor mRNAs in the rat central and peripheral nervous system. J Neurosci 3:1912-1939.

Zoli M, Moretti M, Zanardi A, McIntosh JM, Clementi F, Gotti C (2002) Identification of the nicotinic receptor subtypes expressed on dopaminergic terminals in the rat striatum. J Neurosci 22:8785-8789.

VITA

Kia Janelle Jackson was born July 9, 1982 in Richmond, VA. Kia graduated from Richmond Community High School in June, 2000 as the valedictorian of her high school class and the graduating senior with the highest GPA in Richmond Public Schools. She attended North Carolina A&T State University in Greensboro, NC on a full academic scholarship. Kia graduated Summa Cum Laude and obtained her Bachelor of Science degree in Biology in May, 2004. She was accepted to the Department of Pharmacology and Toxicology at Virginia Commonwealth University in August 2004. Kia joined the lab of Dr. M. Imad Damaj in January 2005, where she began her research on the behavioral and post-receptor calcium-dependent mechanisms of nicotine withdrawal.

In addition to her graduate studies, Kia has attended and presented at several local and national conferences, including the Society for Neuroscience and VCU Watt's Day. Kia also served on the e-board of the Pharmacology and Toxicology Student Organization as the Library representative from 2006-2008, and as an active member of the Women in Science Organization at MCV from 2005-2008, serving as treasurer during the 2007-2008 term.

Manuscripts

- **Jackson KJ**, Martin BR, Changeux JP, Damaj MI (2008) Differential role of nicotinic acetylcholine receptor subunits in physical and affective nicotine withdrawal signs. J Pharmacol Exp Ther 325: 302-312.

- **Jackson KJ**, Walters CL, Miles MF, Martin BR, Damaj MI (2008) Characterization of pharmacological and behavioral differences to nicotine in C57Bl/6J and DBA/2J mice. (in review)

- **Jackson KJ,** Martin BR, Damaj MI (2008) Characterication of a nicotine conditioned place aversion mouse model. (in review, Eur J Pharmacol)

- **Jackson KJ**, Martin BR, Damaj MI (2008) The role of post-receptor calcium-dependent mechanisms in physical and affective nicotine withdrawal behaviors. (in preparation, J Pharmacol Exp Ther)

- **Jackson KJ** and Damaj MI (2008) Nicotine withdrawal-induced decreases in CaMKII and synapsin I function are mediated through β2-containing nAChRs. (in preparation)

- **Jackson KJ**, McIntosh JM, Brunzell DH, Kota DH, Sanjakdar SS, Muldoon PP, Martin BR, Damaj MI (2008) The α6 nicotinic acetylcholine receptor subunit is involved in the behavioral effects of nicotine, morphine, and cocaine. (in preparation, Neuropsychopharmacology)

Abstracts

Jackson KJ, Walters CL, Damaj MI. Role of calcium mediated signaling in the mesolimbic dopamine reward pathway after nicotine reward and sensitization. *Society for Neuroscience*, October 2005

Jackson KJ, Martin BR, Damaj MI. Assessment of the roles of various nicotinic acetylcholine receptor subtypes involved in nicotine withdrawal using transgenic mice. *Society for Neuroscience*, October 2006

Jackson KJ, Martin BR, Damaj MI. Assessment of the roles of various nicotinic acetylcholine receptor subtypes involved in nicotine withdrawal using transgenic mice. *Watts Day*, Virginia Commonwealth University, October 2006

Jackson KJ, Martin BR, Damaj MI. Differential role of nicotinic acetylcholine receptor subunits in physical and affective nicotine withdrawal signs. *Watts Day*, Virginia Commonwealth University, October 2007

Jackson KJ, Martin BR, Damaj MI. The role of post-receptor calcium-dependent mechanisms in physical and affective signs of nicotine withdrawal. *Society for Neuroscience*, November 2007

Jackson KJ, McIntosh JM, Martin BR, Damaj MI. Differential role of α6 and α5 nicotinic receptors in nicotine reward and withdrawal. *Society for Neuroscience*, November 2008

Teaching Experience

Gender/Sex issues in drug abuse. *Drug Dependence PHTX 548*. Fall, 2007