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Investigating the Modulation and Mechanisms of α7 Nicotinic Acetylcholine Receptors in Nicotine Dependence

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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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
2-AG	2-arachidonylglycerol
AChBP	acetylcholine binding protein
AEA	anandamide
Ago-PAMs	agonist-positive allosteric modulators
AMPA	α-Amino-3-Hydroxy-5-Methyl-4-Isoxazole Propionic Acid
CA2+	calcium
CB	cannabinoid
CeA	central nucleus of the amygdala
CPA	conditioned place aversion
CPP	conditioned place preference
CREB	cyclic AMP response element-binding protein
DOR	delta opioid receptor
ERK	extracellular receptor kinase
FAAH	fatty acid amide hydrolase
G proteins	Guanosine triphosphate -binding proteins
GABA	glutamate, γ- aminobutyric acid
ICSS	intracranial self-stimulation
i.p.	intraperitoneal
IPN	interpeduncular nucleus
JAK2	Janus kinase 2
KO	knockout
KOR	kappa opioid receptor
LDT	laterodorsal tegmentum
LHb	lateral habenula
MAGL	monoacylglycerol lipase
MAPK	mitogen-activated protein kinase
MHb	medial habenula
MLA	methyllycaconitine
MOR	mu opioid receptor
MP	minipump
NA+	sodium
NAc	nucleus accumbens
nAChRs	nicotinic acetylcholine receptors
NAAA	N-acylethanolamine hydrolyzing acid amidase
NIH	novelty-induced hypophagia
NMDA	N-methyl-D-aspartic acid
OEA	oleoylethanolamide
PAMs	positive allosteric modulators
PDT	pedunculopontine tegmentum
PEA	palmitoylethanolamide
PFC	prefrontal cortex
PPARα	peroxisome proliferator-activated receptor type-α
s.c.	subcutaneous

SPPARMs	selective PPARα modulators
STAT	signal transducer and activator of transcription
THC	delta 9-tetrahydrocannabinol
VTA	ventral tegmental area
WT	wild type

ABSTRACT

INVESTIGATING THE MODULATION AND MECHANISMS OF α7 NICOTINIC ACETYLCHOLINE RECEPTORS IN NICOTINE DEPENDENCE

By Asti B. Jackson

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

Virginia Commonwealth University, 2017

Major Director: M. Imad Damaj, PhD, Professor, Pharmacology and Toxicology

Tobacco dependence dramatically increases health burdens and financial costs. Limitations of current smoking cessation therapies indicate the need for improved molecular targets. Nicotine, the main addictive component of tobacco, exerts its dependency effects via nicotinic acetylcholine receptors (nAChRs). The homomeric α7 nAChR is one of the most abundant receptors found in the brain and has unique features in comparison to other nAChR subtypes such as high calcium permeability, low probability of channel opening, and a rapid desensitization rate. α7 nAChR agonists reduce nicotine's rewarding properties in the conditioned place preference (CPP) test and i.v. self-administration. Recently, the peroxisome proliferator-activated receptor type-α (PPARα) has been implicated as a downstream signaling target of the α7 nAChR in ventral tegmental area dopamine cells. It is unknown whether the intrinsic characteristics of the α7 nAChR and PPARα are involved in its attenuation of nicotine reward. Therefore, this dissertation sought to investigate the role of α7 nAChRs in a mouse

model of nicotine CPP and nicotine withdrawal by 1) investigating the impact of pharmacological modulation of α7 nAChR function in nicotine dependence and 2) evaluating a possible role for PPARα as a downstream mediator of α7 nAChRs in nicotine dependence. Positive allosteric modulators (PAMs) and a silent agonist were used to investigate the role of α7 nAChR conformations. The utilization of the α7 nAChR Type I PAM NS1738, Type II PAM PNU120596, and silent agonist NS6740 provided insight about the probability of channel opening (NS1738, PNU120596), desensitization (PNU120596, NS6740), and modulation of the endogenous acetylcholine/ choline tone (NS1738, PNU120596) as it relates to the α7 nAChR in nicotine CPP and withdrawal. In addition, this dissertation sought to elucidate the role of the α7 nAChR and PPARα in nicotine dependence using pharmacological interventions. The results suggest that the role of the α7 nAChR in nicotine dependence is conformation-dependent and PPARα-mediated. This dissertation is the first to report PPARα-mediation of the effects of α7 nAChR in nicotine reward and attenuation of nicotine withdrawal signs by PPARα activation. This data supports the development of α7 nAChR agonists and PPARα activators as possible smoking cessation aids.

CHAPTER ONE

GENERAL INTRODUCTION

A. Nicotine Dependence

Tobacco dependence remains one of the leading sources of preventable death worldwide [1,2]. In the United States alone, approximately 550,000 deaths are caused by smoking-related diseases such as cardiovascular disease, chronic obstructive pulmonary disease, diabetes and 12 types of cancers [3]. In particular, it is estimated that 80% of lung cancer cases are caused by smoking [4]. The economic burden of smoking is over $280 billion dollars annually (including smoking-related health costs and productivity losses)[5]. Although the rate of smoking has declined (20.9% in 2005 to 15.1% in 2015)[6], there are still about 40 million individuals who engage in tobacco use in the United States[7]. There are possible explanations that can account for this continued tobacco use. The perpetuation of tobacco use may be due to the switching of traditional tobacco products such as cigars and cigarettes to smokeless tobacco products, hookah and e-cigarettes[8]. This transition to newer tobacco products is thought to be driven by the reduced harm perception of these products compared to cigarettes [9]. Due to the limited scientific evidence available, it is unclear whether e-cigarettes have any long-term harmful effects or can act as smoking cessation treatment [10]. In addition, there is a growing concern that e-cigarette use may normalize smoking behaviors and promote the use of traditional tobacco products[11]. This is even more alarming since e-cigarette use has doubled in adolescents in recent years[8]. Smoking initiation during adolescence is another factor that may sustain tobacco-smoking rates nationally. Adolescence is a unique period marked by considerable neurobiological changes[12,13] risking taking behavior[14] and experimentation with drugs of abuse including tobacco products[5]. In addition, drug use

during adolescence is a predictor for substance abuse in adulthood. It is estimated that 90% of adult smokers have reported having their first cigarette before age 18 [5]. Another explanation for the continued tobacco use may lie with the modest success rate of current smoking cessation therapies with less than 30% of individuals remaining abstinent for more than 1 year [15]. The current smoking cessation aids (varenicline , bupropion, and nicotine replacement therapies) all share a common mechanism of action by interacting with nicotinic acetylcholine receptors (nAChRs) [16,17]. Varenicline (Chantix®) is marketed as a high affinity α4β2* (* denotes the inclusion of other subunits in the receptor) nAChR partial agonist with other targets including the α7 nAChRs and α3β4* nAChRs where it acts as a full agonist[18,19]. Bupropion is an FDA-approved antidepressant marketed under the name Wellbutrin XL® and is also indicated as a smoking cessation aid (Zyban®)[20]. Its mechanisms of action include dopamine reuptake inhibitor [21] and noncompetitive antagonist of α3β2, α4β2*, and α7 nAChRs[22–25]. Nicotine replacement therapies such as the nicotine patch (NicoDerm CQ®) partially replace nicotine at nAChRs in an attempt to relieve withdrawal symptoms[26]. The modest efficacy of the current smoking cessation aids raises the need for a better understanding of the complex neurobiology underlying nicotine dependence. This in turn will aid in the discovery of new molecular targets and the development of more effective treatments.

B. Nicotine and Nicotinic Acetylcholine Receptors

Cigarette smoke has over 4,000 components[27]; however, nicotine is thought to primarily mediate the rewarding effects of tobacco. Nicotine has been shown to have reinforcing and positive subjective effects in humans [28,29]. Nicotine is also self-administered in rodents[30–32] and non-human primates[33,34] and induces a preference in the conditioned place preference test[35–37].

Nicotine mediates its effects through nAChRs[38] which belong to the Cys-loop receptor family and are ligand gated ion channels that form pentamers arranged around a water-filled pore[39,40]. The subunits of mammalian neuronal nAChRs range from α2-α7, α9, α10 and β2-β4. These receptors are permeable to both Na^+ and Ca^{2+} and can form homomeric and heteromeric receptors[41]. Nicotinic subunits can assemble in different combinations resulting in a diversity of functions of nAChR subtypes. These receptors have three broad conformational states: resting closed states, open states, and desensitized states [42]. The typical resting closed state is induced when the orthosteric site (traditional ligand binding site) is unoccupied and the cation channel is closed. Upon binding of an orthosteric agonist, the cation channel is opened which allows the influx of cations into the cell. Following the open state the receptor is then desensitized; despite agonist binding the cation channel is closed rendering the receptor inactive[43]. However, there are new compounds known as "silent agonists" that do not behave as typical orthosteric agonists. Silent agonists are orthosteric agonists that do not cause channel opening after binding, but instead promote conformational changes associated with the desensitized state[44–46]. nAChRs are located pre-, post and extrasynaptically throughout the central nervous system [47] where they aid in fast synaptic transmission and modulation of neurotransmitter release [48]. The most abundant nicotinic receptors found in the mammalian brain are the nicotinic low affinity homomeric α7 and the nicotinic high affinity heteromeric α4β2*[49]. These two classes of nAChRs have diverse characteristics. The α7 nAChR has high calcium permeability, low probability of opening, rapid desensitization (in milliseconds) and binds α-bungarotoxin[50,51]. In contrast, the α4β2* nAChR has a high probability of opening, desensitizes at a slower rate (in seconds) and does not bind α-bungarotoxin[52]. nAChRs, like most proteins, have orthosteric binding sites (traditional agonist binding sites) and allosteric binding sites (nontraditional agonist binding sites)[53,54]. This has

allowed for the development of pharmacological tools that can induce activation of nAChRs via various mechanisms. Positive allosteric modulators (PAMs) bind to the allosteric site of nAChRs and enhance the efficacy of endogenous agonists (acetylcholine and choline) and the probability of channel opening, decrease the rate of desensitization, and increase the affinity of ligands without having an effect on their own[42,55,56]. Pharmacological interventions along with preclinical animal models of nicotine reward and withdrawal will further the understanding of the underlying mechanisms of nicotine dependence.

C. Preclinical Models for Measuring Nicotine Dependence

Animal models are invaluable to drug abuse research. Research conducted with animal subjects can be controlled for variables and allow for thorough investigation of underlying mechanisms[57]. There are multiple models used to assess various aspects of nicotine dependence in rodents and nonhuman primates such as reward, reinforcement and withdrawal. Self-administration is a model of drug reinforcement that is thought to mimic drug seeking and drug taking behavior in humans [58]. With the exception of hallucinogens, drugs that are abused in humans are typically self-administered in animal models given it a high degree of face validity and predictive validity [59]. Nicotine self-administration has even been demonstrated in humans in a laboratory setting [28]. In this operant conditioning paradigm nonhuman subjects range from monkeys to rodents and the typical drug reinforced behaviors include lever presses and nose pokes for rodents and a panel press response for nonhuman primates[57]. The delivery of the drug can vary from oral, intramuscular, and most commonly via intravenous catheterization. In the case of nicotine, the primary route of administration in humans is through inhalation which produces a rapid onset of

action; therefore, the most desirable and controlled drug delivery method that allows for a rapid onset for nicotine self-administration in rodents is intravenous catheterization[32].

Drug discrimination is a paradigm that classifies and categorizes drugs based on their interoceptive effects [60]. Commonly abused drugs in humans produce interoceptive effects that may contribute to their abuse liability. Abused drugs that produce discriminative effects in animals produce subjective effects in humans [61,62] including nicotine [63]. However, drugs that have no abuse liability, such as the atypical antipsychotic drug clozapine [64], can produce discriminative stimulus effects [60]. Drug discrimination has predictive validity for CNS-mediated compounds [65] . This technique consists of a food reinforced operant response of a lever press or nose poke in the case of rodents. During training sessions, rodents are pretreated with drug or vehicle and the correct lever press results in food pellet presentation. Drug discrimination investigates whether other drugs produce similar interoceptive effects as the training drug or whether another compound can augment the interoceptive effects of the training drug [66].

Intracranial self-stimulation (ICSS) is a model of operant conditioning that measures abuse liability of drugs. A monopolar or bipolar electrode is implanted in brain regions such as the medial forebrain bundle. Medial forebrain bundle excitation produces stimulation of the mesolimbic pathway (pathway associated with reward)[67].The electrical stimulation from the electrode reinforces a behavioral response such as lever presses in rodents[68]. The frequency or amplitude of electrical stimulation can be manipulated. Drugs of abuse are said to 'facilitate' ICSS if the drug causes a leftward shift of ICSS stimulation frequency-rate curves and decrease ICSS thresholds [69]. Nicotine along with other drugs of abuse facilitate ICSS stimulation [70]. Drug-induced ICSS facilitation is thought to correlate with drug abuse potential in humans giving this model predictive validity [68].

Conditioned place preference (CPP) is a Pavlovian conditioning paradigm used to asses drug reward [71]. CPP involves associative learning where animals are thought to pair the rewarding effects of a drug (unconditioned stimulus) with the context the drug was once received (conditioned stimulus). This drug-induced association is clinically relevant. It has been reported that exposure to drug-related cues in dependent users induces drug cravings [72]. In particular, smoking cues such as a burning cigarette or a lighter associates with rewarding effects induced by nicotine which perpetuates smoking behavior in humans[73,74]. Smoking cues not only induce cravings that can reinforce smoking but also induce physiological responses such as increased blood pressure and heart rate [75,76]. Drugs abused in humans induce a preference in the CPP test in animal models giving the model predictive validity. The CPP test has also been performed in humans[77].

a. Conditioned Place Preference Methods

Our lab uses an unbiased, counterbalanced and randomized CPP protocol. In the typical CPP test, there are a set of distinct contextual cues. Our CPP apparatus has three chambers in a linear arrangement. The white external chamber (visual cue) consists of a mesh floor texture (tactile cue) and the black external chamber (visual cue) has a rod floor texture (tactile cue). The external chambers are separated by a smaller gray chamber with a smooth PVC floor. Mice are then conditioned with drug or vehicle in the white or black chambers. On baseline day mice are free to roam all three chambers, the time spent in the white, and black chambers are recorded. On conditioning days after drug injection mice are confined to one compartment for 20 min and 4 hrs. later they were confined to the other compartment with the injection they did not receive in the morning session (be that vehicle or drug). On test day, mice are allowed access to all chambers for 15 min in a drug free state. The preference score was calculated by determining the

difference between the time spent in the drug paired side during test day versus the time in drug-paired side during the baseline day. The nicotine CPP paradigm has been well established by our lab and others [36,37,78,79]. Nicotine has a narrow dose response curve in the CPP test and the dose of 0.5mg/kg of nicotine that is typically used in our studies has been shown to induce a significant preference in mice in the CPP test[78,80]. CPP has some limitations that could be considered potential confounding factors for the interpretation of the results (locomotor activity changes, novelty-seeking behavior on test day, and contextual preferences for one side or the other). To address the potential effect that drugs may have on locomotor activity, our CPP boxes are equipped with infrared beams that measure the locomotor activity of animals during the test. During the test day, animals are in a drug free state and there are typically no differences observed of locomotion between treatment groups. Also, mice naturally explore novel areas or objects [81]. To address this possible confound our boxes are 3-chamber compartments (with a central compartment), which limits the impact of novelty-seeking behavior on test day. The most novel chamber is the center chamber that is not paired with drug or vehicle. Mice are only exposed to this chamber on baseline and test day whereas they are exposed to the other chambers throughout the duration of the experiment. In addition, our extensive work with mice on an ICR background over the years in the CPP test showed that this propensity for contextual preference is rare in this strain, and any mouse showing preference for one side higher than 65% on the baseline day was not used in the study.

Nicotine withdrawal is one aspect of nicotine dependence that is considered to be a negative reinforcer for perpetuating tobacco use [29]. The current smoking cessation therapies are thought to attenuate this important component of nicotine dependence[82]. Nicotine withdrawal symptoms in humans consist of physical signs (bradycardia, gastrointestinal discomfort, increased appetite),

cognitive signs (difficulty concentrating, impaired memory), and affective signs (anxiety, depressed mood, anhedonia) [83,84]. Rodents serve as a model to investigate nicotine withdrawal. To mimic human nicotine exposure, rodents receive chronic nicotine via various routes of administration such as orally [85,86], intravenous infusion [87], subcutaneous (s.c.) minipump (MP) [88–90], and chronic systemic injections [91,92]. Nicotine withdrawal is induced either spontaneously (removal of chronic nicotine) or precipitated via the administration of nAChR antagonists such as the nonselective nAChR antagonist mecamylamine. Physical signs of nicotine withdrawal assessed in rodents are hyperalgesia [85,93], somatic signs, such as paw tremors, body tremors, grooming, and backing [89,94] and alterations in locomotor activity [95]. Cognitive signs induced by nicotine withdrawal in rodents manifest as deficits in the number of reversals, increased omissions, and reduced speed of responding in the probabilistic reversal learning task[96]. Affective signs of nicotine withdrawal are anxiety-like behaviors as measured in the elevated plus maze test and light-dark boxes [97,98], dysphoric-related behaviors in the conditioned place aversion (CPA) test [99,100], and anhedonia as observed with elevated reward thresholds in ICSS [101,102]. Current smoking cessation therapies are thought to target the nicotine withdrawal syndrome in humans and are effective in preclinical models of nicotine withdrawal attributing predictive validity to these models. Varenicline and bupropion reduce cognitive deficits [103,104] , bupropion attenuates somatic and affective signs [105,106] and nicotine replacement reverses physical, affective, and cognitive signs [89,107] associated with the nicotine withdrawal syndrome in rodents.

b. Nicotine Withdrawal Methods

In our lab, mice receive chronic nicotine via s.c. osmotic MPs that are implanted under isoflurane anesthesia. Nicotine (24mg/kg/day) or saline is infused for 14 days and the concentration of nicotine is adjusted according to animal weight and mini pump flow rate. On the morning of day 15, mice are injected with vehicle or test drug before the challenge with the nAChR antagonist, mecamylamine (2 mg/kg, s.c.). Withdrawal is assessed 10 min after mecamylamine administration. Affective (anxiety-like behavior) and physical (somatic signs, hyperalgesia) signs of nicotine withdrawal are evaluated in this paradigm. Anxiety-related behavior is measure in the elevated plus maze test for 5 minutes. Time spent on the open arms of the plus maze is assessed as a measure of anxiety-related response. The number of arm crosses between the open and closed arms are counted as a measure of locomotor activity. Somatic signs are assessed immediately following the plus maze test for 20 min. Somatic signs are measured as paw and body tremors, head shakes, backing, jumps, curls, and ptosis. Mice are placed in clear activity cages without bedding for the observation period. The total number of somatic signs is tallied for each mouse and the average number of somatic signs during the observation period is plotted for each test group. Hyperalgesia is evaluated using the hot plate test immediately following the somatic sign observation period. Mice are placed into a 10-cm wide glass cylinder on a hot plate (Thermojust Apparatus, Richmond, VA) maintained at 52°C. The latency to reaction time (jumping or paw licking) is recorded. The specific testing sequence was chosen based on our prior studies showing that this order of testing reduced within-group variability and

produced the most consistent results [93]. An observer blinded to experimental treatment performs all studies.

D. Mechanisms Underlying Nicotine Reward

Nicotine initiates its rewarding effects by activating the natural reward system of the brain known as the mesolimbic pathway. This pathway is comprised of dopaminergic neurons originating in the ventral tegmental area (VTA) that project to regions such as the nucleus accumbens (NAc), prefrontal cortex (PFC), amygdala and hippocampus [108–110]. Dopamine release, especially in the NAc, is associated with the rewarding and reinforcing effects of all drugs of abuse [111].There have been many studies implicating this pathway in nicotine reward. Blockade of dopamine receptors or 6-hydroxydopamine lesions in the mesolimbic pathway results in a decrease in nicotine reward-like behavior in several preclinical tests such as self-administration, CPP and ICSS [112,113]. Infusion of nicotinic antagonists directly in the VTA attenuates nicotine self-administration[114]. Nicotine increases dopamine neuron firing rate and dopamine release in areas of the brain such as the NAc shell, extended amygdala and PFC [108,112,115,116] via nAChRs [117]. This pathway has a complex circuitry that also involves other neurotransmitters such as glutamate, γ-aminobutyric acid (GABA), acetylcholine, endocannabinoids, and opiod peptides. Glutamatergic, GABAergic, and cholinergic inputs converge on dopamine neurons modulating dopamine release [118].

The excitatory neurotransmitter glutamate has been implicated in nicotine reward. Systemic administration of glutamate ionotropic receptor antagonists attenuated nicotine-evoked increases of dopamine levels in the NAc[119]. Behaviorally it has been shown that the glutamate N-methyl-D-aspartic acid (NMDA) receptor antagonist LY235959 infused into the VTA and the central

nucleus of the amygdala (CeA) reduces the reinforcing effects of nicotine i.v. self-administration and block nicotine ICSS facilitation in rats [120]. Acute doses of nicotine have been shown to increase glutamate release in the NAc [121,122] . It has been suggested that dopamine release in the NAc is dependent upon NMDA activation in the VTA[123].

An enhancement of the inhibitory neurotransmitter GABA has been shown to reduce the rewarding effects of nicotine. The $GABA_B$ receptor agonist, baclofen, attenuates nicotine-induced dopamine release in the NAc shell and reduces nicotine i.v. self-administration in rats [124,125]. In addition, the effect of baclofen in nicotine self-administration is dependent on $GABA_B$ receptors in the VTA and the pedunculopontine tegmentum (PDT), an area in the brain stem containing cholinergic and glutamatergic neurons [126,127]. Also, the $GABA_B$ receptor PAM BHF177 reduces nicotine self-administration in rats after chronic exposure [128]. This suggests that modulation of the $GABA_B$ receptor is important in nicotine reward.

Cholinergic and glutamatergic neurons in the laterodorsal tegmentum (LDT) and the PDT initiate excitation of dopamine neurons in VTA that project to the NAc [129,130]. Lesions of cholinergic neurons in the PDT reduce nicotine self-administration in rats [131]. nAChRs are located pre and postsynaptically throughout the mesolimbic circuitry [130,132,133]. The utilization of genetically mutant mice, pharmacological interventions, and viral re-expression approaches have implicated particular brain areas and specific nicotinic subtypes involved in nicotine reward. The nicotinic high-affinity β2-containing nAChRs are required for nicotine reward and reinforcement as revealed in nicotine CPP and nicotine i.v. self-administration studies in β2 knockout (KO) mice [37,134,135]. The β2 subunit co-assembles with the α6 and α4 subunits to form several α6β2*, α4β2*,α4α6β2* nAChR subtypes, which have been notably expressed in the midbrain region such as the VTA [136–138]. Nicotine CPP revealed a critical role of the α4, α6, and β2 subunits in

the NAc via genetic mutant mice and site specific infusions[139]. In addition, a genetic ablation of the β2, α6, and α4 nAChR subunits attenuated nicotine self-administration in mice but nicotine self-administration was maintained in KO mice where the analogous subunit was only re-expressed in the VTA via a lentiviral vector [31,135]. Furthermore, in the nicotine CPP test α4 "knock-in" mice (Leu9' Ala mutation renders animals hypersensitive to nicotine) produced a preference for nicotine at a dose 50-fold lower than the typical nicotine dose that induces a preference in wild type (WT) mice [140]. In recent years genome wide association studies in humans revealed a variant in the *CHRNB4/A3/A5* gene cluster (encodes α3, α5, β4 nicotinic subunits), located in chromosome region 15q25, serves as a risk factor for lung cancer and nicotine dependence[141–143]**.** More specifically, a reduction of function of CHRNA5 (D398N) is linked to increased risk for tobacco dependence [144,145]. Indeed, in human pluripotent cells that were induced into midbrain dopaminergic neurons, nAChRs that contained the nonsynonymous human CHRNA5 D398N polymorphism (rs16969968) had a decreased potency of nicotine compared to controls[146]and increased consumption of nicotine in intravenous self-administration in mice[147]. Similarly, α5 KO mice have an increase in nicotine intake in the nicotine intravenous self-administration test and do not display raised brain stimulated thresholds after administration of an aversive dose of nicotine in comparison to their WT counterparts [147,148]. Similar observations occurred in the nicotine CPP paradigm where α5 KO mice exhibited a maintained nicotine preference at higher doses not maintained by α5 WT mice [149]. This suggests that the α5 subunit may act as an inhibitory responder that limits nicotine consumption and rewarding effects [148,149]. α3β4* nAChRs mediate nicotine reward. The α3β4*-selective antagonist AuIB attenuated nicotine preference in α5 WT and KO mice [150] suggesting the α3β4*nAChR influences nicotine reward independent of the α5 subunit. In addition, β4 KO mice had a

reduction in nicotine reinforcement and motivation to self-administer nicotine in the nicotine intravenous self-administration paradigm [151]. However, β4 subunit overexpression in Tabac mice (transgenic mouse model of the *Chrnb4-Chrna3-Chrna5* gene cluster) results in nicotine CPA and a reduction in nicotine consumption [152]. The divergent effects of the β4 subunit in these studies may be the result of different doses of nicotine used and the different aspects of nicotine intake investigated (i.e. reward and aversion).

The nAChRs and cannabinoid (CB) receptors are both expressed in overlapping rewarding brain regions and it has been shown that these two systems interact with each other [153,154]. Genetic deletion of the CB_1 receptor and administration of the CB_1 receptor antagonist rimonabant attenuates nicotine i.v. self-administration and nicotine CPP [155–157]. Conversely, a synthetic CB_1 receptor agonist WIN 55,212-2 enhances nicotine self-administration in rodents [158]. In addition, CB_2 receptors play a role in nicotine reward. Nicotine CPP was abolished in CB_2 KO mice and blocked after administration of the CB_2 antagonist SR144528 [159]. In addition, pharmacological blockade or deletion of fatty acid amide hydrolase (FAAH), the degradative enzyme for the endogenous CB receptor ligand anandamide (AEA), enhances nicotine reward as seen in the nicotine CPP test [155]. This suggests that indirect activation of CB receptors is capable of enhancing nicotine reward.

The opioid system also plays a role in nicotine reward. The endogenous opioid system consists of three receptors: mu (MOR), delta (DOR), and kappa (KOR) opioid receptors [160]. The endogenous peptide β-endorphin binds the MOR with high affinity, met- and leu-enkephalin bind to the DOR, and dynorphins preferentially bind to KORs [161]. The MOR antagonist naloxone attenuated nicotine intravenous self-administration [162] and nicotine CPP [163]. In addition, mice lacking the endogenous MOR agonist β-endorphin and MOR KO mice both showed an

attenuation of nicotine CPP [163,164]. This suggests that the MOR may mediate nicotine reward and reinforcement. Pharmacological blockade and genetic deletion of the DOR attenuates nicotine CPP and self-administration as well [165]. In contrast, activation of KORs attenuate nicotine self-administration [162], which supports its involvement in emotional states.

E. Mechanisms Underlying Nicotine Withdrawal

Reward systems in the brain undergo neuroadaptations after chronic exposure to nicotine in tobacco products, which leads to nicotine dependence. Cessation from cigarette smoking induces a withdrawal syndrome comprised of physical, affective and cognitive symptoms. The severity of these symptoms is a risk factor for relapse [29,166]. Therefore, understanding the mechanisms involved in nicotine withdrawal may aid in the production of more successful smoking cessation therapies. Neuroadaptations caused by nicotine withdrawal involve neurotransmitter systems that are also involved in nicotine reward: glutamate, GABA, dopamine, endocannabinoid, and opioid systems [154,167].

There is evidence to suggest that glutamate plays a role in the affective and somatic signs produced by nicotine withdrawal in rodents. It has also been shown that glutamate release and NMDA activation is necessary for the manifestation of somatic signs in nicotine withdrawn mice [168]. Nicotine withdrawal-induced elevations of brain reward thresholds in ICSS are interpreted as depression-like behavior [169]. Similar to nicotinic antagonists, antagonism of the α-Amino-3-Hydroxy-5-Methyl-4-Isoxazole Propionic Acid (AMPA) glutamatergic receptor results in this brain reward threshold elevation in nicotine-dependent rats[170]. Furthermore, activation of glutamatergic autoreceptors produced elevation in reward thresholds[170]. This suggests that a

reduction in glutamatergic transmission may possibly be responsible for the affective signs induced by nicotine withdrawal. However, genetic deletion of the metabotropic glutamate receptor 5 in mice attenuated the affective signs associated with nicotine withdrawal [101]. Taken altogether, the glutamate system plays a role in the affective signs of withdrawal but different glutamate receptor classifications may have divergent effects.

There is evidence to suggest that GABA neurotransmission plays a role in nicotine withdrawal. Mice that lack the $GABA_B$ receptor exhibited attenuated somatic signs [171]. In addition, GABAergic neurons in the interpeduncular nucleus (IPN) are activated during nicotine withdrawal and attenuating the excitability of these neurons was shown to alleviate nicotine withdrawal somatic signs in mice [168]. However, administration of $GABA_B$ receptor agonist, PAM, and antagonist all elicited an exacerbation of depressive-like behavior as indicative of elevated brain reward thresholds in ICSS [172]. Further studies are needed to provide clarity for the role of GABA in the affective signs induced by nicotine withdrawal.

Nicotine withdrawal is thought to produce a hypodopaminergic state evidenced by decreased dopamine levels in the NAc of rats [173,174], reduction in dopamine release in the NAc [175], and brain reward deficits [169]. KOR signaling may play a part in inducing this hypofunctional dopaminergic state. KOR signaling has been associated with mood and depressive-like states [176,177]. KOR activation decreases dopamine levels in the NAc [178] by blocking dopamine release and enhancing dopamine reuptake [179,180]. This has sparked interest in its involvement in nicotine withdrawal, especially the affective signs. Indeed, KOR antagonists nor-BNI, JDTic, and LY2456302 alleviated the physical and affective signs of the nicotine withdrawal syndrome in rodents [97,149,181].

nAChRs are the predominate mediator of nicotine withdrawal symptoms. The nonselective nAChR antagonist mecamylamine is known to precipitate nicotine withdrawal signs in nicotine-dependent rodents [89,93,94,182]. Pharmacological interventions and mouse KO studies revealed that nicotinic receptor subunits modulate different aspects of the nicotine withdrawal syndrome. The differential expression and pharmacological profiles of nAChR subtypes may account for their various involvement in nicotine withdrawal. The affective signs are primarily mediated by the β2 [93,183] α6 [184] β4 [185], and α7 [185] as indicated in the elevated plus maze test, CPA and ICSS. The physical signs of the nicotine withdrawal syndrome are mediated by α3[150] α5 [93,186], α2 [186], β4 [150,185] and a subset are mediated by α7 subunits [93,187]. One interesting feature of chronic nicotine exposure is the upregulation of nAChRs, most notably α4β2*[188]. This phenomenon has been observed *in vitro* [189],in preclinical animal studies [190,191] and in humans [192]. Upregulation of nAChRs after chronic administration may be in response to receptor desensitization to compensate for receptors no longer responding to agonist activation; however, it is unknown whether or not the upregulated receptors are functional [193]. Interestingly, rodent and human studies suggest a positive correlation of nicotine withdrawal signs with upregulation of α4β2*nAChRs [194,195].

Recently, neural circuitry such as the habenulo-interpeduncular pathway has been implicated in nicotine withdrawal and aversion [148,186]. The habenula is subdivided into two regions: medial habenula (MHb) and the lateral habenula (LHb)[196]. The MHb is predominately thought to play a role in nicotine dependence and it has afferents that project to the IPN [197]. nAChRs are densely expressed in the MHb-IPN pathway [198]. Indeed, microinjection of the nonselective nAChR antagonist mecamylamine into the MHb or the IPN precipitated nicotine withdrawal in mice [186]. In particular, blockade of the β4 subunit in the IPN induced nicotine withdrawal-induced somatic

signs in mice[168]. In addition, infusion of the α6* nAChR-selective antagonist α-conotoxin MII in the MHb attenuated anxiety-like behavior in nicotine withdrawn mice [199].

The endocannabinoid system has also been implicated in nicotine withdrawal. Activation of CB_1 receptors with delta 9-tetrahydrocannabinol (THC) has been shown to reduce the physical signs of withdrawal in rodents [200]. FAAH KO mice and pharmacological inhibition of FAAH results in an increase level of the endocannabinoid AEA [201]. AEA is an endogenous agonist at the CB_1 receptor; therefore, blockade of FAAH is thought to indirectly activate CB_1 receptors. Contrary to the effect of THC on nicotine withdrawal induced somatic signs, pharmacological and genetic blockade of FAAH resulted in exacerbated somatic signs [155]. Also, CB_1 genetic ablation did not affect nicotine withdrawal-induced somatic signs [200]. The lack of effect on nicotine withdrawal in CB_1 KO mice could be the result of compensatory effects because the CB_1 receptor antagonist rimonabant attenuates somatic signs in nicotine withdrawn mice [155]. In addition, there is a report to suggest fluctuations in AEA levels in nicotine withdrawn rats [202]. Even though the levels of the endocannabinoid 2-arachidonylglycerol (2-AG) were unchanged in nicotine withdrawn rats [202], monoacylglycerol lipase (MAGL), enzyme responsible for the degradation of 2-AG, KO mice exhibited attenuated nicotine withdrawal- induced somatic signs and administration of the MAGL inhibitor, JZL184, reduced somatic and affective withdrawal signs in a CB_1-dependent manner [182]. CB_2 KO mice did not produce altered nicotine withdrawal signs compared to their WT counterparts [159] while another study suggested that CB_2 KO mice had an attenuation of somatic signs [203]. The genetic backgrounds of the mice used in the studies were different and may account for the divergent effect observed in nicotine withdrawal. Taken together, more investigation is warranted to understand the role of the endocannabinoid system in nicotine withdrawal.

F. α7 nAChR Physiological and Pharmacological Properties

Many potential targets and neurotransmitter systems involved in the various aspects of nicotine dependence have been discussed above. These neurotransmitter systems are important, but nAChRs are the primary targets of nicotine. Thus, this dissertation will primarily focus on the nAChRs of the cholinergic system. There are two abundant nicotinic subtypes found in the brain, β2* and α7 nAChRs[49]. However, the role of the α7 nAChR is understudied in nicotine dependence in comparison to β2* nAChRs. β2* nAChRs have been the primary focus of nicotine dependence research. It has been shown that low nicotine levels that smokers are exposed to occupy the majority of high affinity β2* nAChRs in the brain [204,205]. These receptors are also upregulated in postmortem brains of smokers [206] and animals [207] who received nicotine chronically. In addition, preclinical studies showed that the β2 subunit is required for nicotine reward, reinforcement, and some aspects of withdrawal [93,134,135,183]. However, given the ability of the β2 subunit to co-assemble with multiple subunits forming various nicotinic receptor subtypes with different pharmacological and expression profiles, it has become arduous to identify which β2* nAChR subtypes are involved in nicotine dependence. In addition, β2-targeting smoking cessation aids such as varenicline and nicotine replacement therapies have modest efficacy. Thus, it is important to investigate other molecular targets. The other most abundant nicotinic receptor found in the brain, α7 nAChR, is found in areas related to reward such as the hippocampus, amygdala, VTA, NAc, and IPN [41,122,208]. In addition, the α7 nAChR has unique characteristics that set it apart from other nAChR subtypes. The structure of the α7 nAChR shares a high homology with the acetylcholine binding protein (AChBP) found in snails [209]. The α7 nAChR is made up of five identical α7 subunits creating five potential binding sites between

the interfaces in contrast to the heteromeric $(\alpha4)_2(\beta2)_3$ nAChRs with only two binding sites [210,211]. There has been evidence to suggest that the α7 subunits can co-assemble with β2 subunits forming a heteromeric receptor with the following combinations: $(\alpha7)_3(\beta2)_2$ and $(\alpha7)_4(\beta2)_1$ [212–215]. However, the implications and function of this receptor subtype in the mammalian brain is not well understood. All nAChRs are permeable to cations such as Na^+ and Ca^{2+}; however, α7 nAChRs favor Ca^{2+} influx over Na^+ in a ratio of 10:1 [216,217] which is a critical feature for its role in neurotransmitter release. α7 nAChRs located on presynaptic mesolimbic neurons function as modulators of neurotransmitter release [48]. Activation of presynaptic α7 nAChRs on glutamatergic terminals in the VTA [218], modulate glutamate release that activates dopaminergic neurons and causes dopamine release in the NAc [218,219]. In addition, α7 nAChRs are found on glutamatergic terminals in the VTA that synapse to GABAergic neurons [220] that upon activation inhibit dopamine neurons. In the NAc, α7 nAChRs on glutamatergic afferents that synapse to medium spiny neurons can potentiate glutamate release and concomitantly activate ionotropic glutamate receptors on dopaminergic axon terminals [221,222] inducing dopamine release. Furthermore, preterminal α7 nAChRs on glutamatergic terminals in the NAc can also induce metabotropic glutamate receptor activation on dopaminergic terminals, resulting in an attenuation of dopamine release [223]. A depiction of the neurocircuitry of α7 nAChRs in the VTA and NAc can be found in Fig.1. In the PFC preterminal α7 nAChRs on glutamatergic terminals induce dopamine release in this brain region via involvement of ionotropic glutamate receptors on dopaminergic terminals [224,225]. α7 nAChRs are also located post and extrasynaptically in brain areas such as the hippocampus, VTA and PFC where they are thought to aid in traditional fast synaptic transmission [218,226–228]. Postsynaptic α7 nAChRs in the CA1 region of the hippocampus are involved in the induction of long term potentiation [229] In contrast to β2*

nAChRs, α7 nAChRs have a low probability of being open and are profoundly desensitized in the presence of high agonist concentrations [230]. The desensitization of the α7 nAChRs alters it function throughout the neurocircuitry and may lead to different net outcomes on neurotransmitter release.

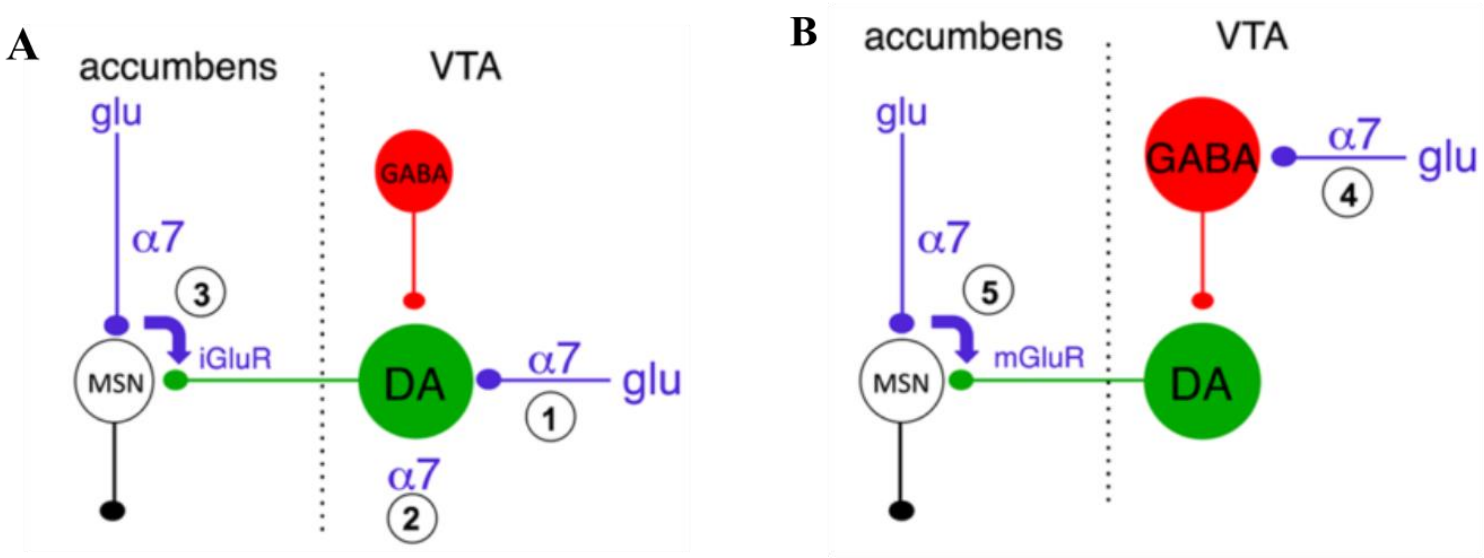


Figure 1: Schematic of α7 nAChR Neurocircuitry in the VTA and NAc *(Adapted from* [231])

A: (1) α7 nAChRs located on glutamatergic terminals synapse onto dopaminergic neurons in the VTA. (2) Somatodendritic α7 nAChRs are located on dopamine neurons in the VTA. (3) Preterminal α7 nAChRs on glutamatergic afferents synapse with medium spiny neurons and glutamate release stimulates ionotropic glutamate receptors on dopaminergic neurons in the NAc. **B**: (4) Glutamatergic terminals in the VTA possess α7 nAChRs. The glutamatergic afferents synapse onto GABAergic neurons and they inhibit dopamine neurons. (5) In the NAc, presynaptic α7 nAChRs are located on glutamatergic terminals. They synapse onto medium spiny neurons and glutamate release activates metabotropic glutamate receptors on dopaminergic terminals.

G. Conformational Regulation of α7 nAChRs by Pharmacological Interventions

The conformational changes of the α7 nAChR may play an important role in its pharmacological and molecular effects in different disease states. The α7 nAChR is an allosteric protein with orthosteric (traditional) binding sites and allosteric binding sites. Activation of the α7 nAChR which an orthosteric agonist is known to produce intrinsically limiting factors such as a low probability of opening and a rapid desensitization rate [51]. To circumvent these limitations and/or understand the effects of desensitization and enhanced channel opening in different behavioral responses, several types of allosteric modulators of α7 nAChRs were developed. For example, PAMs bind to allosteric sites most likely in the transmembrane domain of the receptors [232,233] and increase the effectiveness of an orthosteric agonist. The presence of an orthosteric agonist is required for activation to occur. In comparison to orthosteric agonists, PAMs modulate the endogenous tone and restrict activation to only where acetylcholine is released and choline is present [234]. PAMs are broadly classified into two groups: Type I PAMs and Type II PAMs. Type I PAMs, such as NS1738, increase the probability of opening of α7 nAChRs by attenuating the energy barriers that prevent transitions to the active state of the receptor. In contrast, Type II PAMs, such as PNU120596, not only increase the opening probability, but alter the equilibrium of the receptor in such a way that the active state is favored over the desensitized state resulting in prolonged opening [235,236]. Both PAMs increase the probability of channel opening and thus increase channel conductance; however, this is a sole feature attributed to Type 1 PAMs. Therefore, Type I PAMs can serve as pharmacological tools to investigate the effect of enhanced channel conductance of the α7 nAChRs. Type II PAMs not only increase the probability of opening but also reduce the desensitization rate. They can also reactivate receptors that are desensitized [42]. These pharmacological tools can be used to identify the role of α7 nAChR

desensitization rate especially if both categories of PAMs are used in the same studies along with an orthosteric agonist. PAMs could also provide more selectivity for α7 nAChR activation since α7 nAChRs and serotonin 5-HT_3 receptors have a high homology of their ligand binding domains [237]. Both categories of PAMs were shown to have potential procognitive properties [238,239], and anti-inflammatory and anti-allodynic effects in rodents [240,241].

Ligands known as dual allosteric agonist-PAMs (Ago-PAMs) were reported *in vitro* to have both agonist and PAM properties [242]. The Ago-PAM GAT107 is the active isomer of the Type II α7 nAChR PAM TQS and is thought to bind to the same site as PNU120596 to induce its allosteric modulation effects. GAT107 does not bind to the orthosteric site to induce its direct receptor activation but another distinct allosteric site. The orthosteric site does not need to be occupied for GAT107 to induce its effect [243]. The Ago-PAMs may be used to understand α7 nAChR activation independent of the orthosteric site. GAT107 has been shown to reduce inflammatory and neuropathic pain in rodents [244].

The recent emergence of silent agonists for the α7 nAChR, such as NS6740, represents an interesting and new approach to modulate α7 nAChR subtypes. α7 nAChR silent agonists are high affinity ligands that bind to the orthosteric binding site but possess very low efficacy (<2-3%) [245]. They are considered "desensitizers" that bind to α7 nAChRs and induce conformational changes that favor the desensitization state over the active state [246]. The agonist properties of the silent agonist are revealed once co-applied with a type II PAM [44] suggesting that it acts as a typical agonist after the destabilization of desensitization. α7 nAChR silent agonists can serve as pharmacological tools to assess the effect of α7 nAChR desensitization/ lack of conductance in disease states. For example, while NS6740 was ineffective in rodent cognition assays [245], it has shown analgesic-like properties in chronic pain models [246], suggesting that there may be a

necessity of ion conductance/ desensitization of the α7 nAChR for CNS-related behavioral effects.

The pharmacological effect of these new α7 nAChR ligands is unknown in preclinical nicotine dependence models. The utilization of these ligands could implicate distinct conformations of the α7 nAChR that are necessary for certain aspects of nicotine dependence.

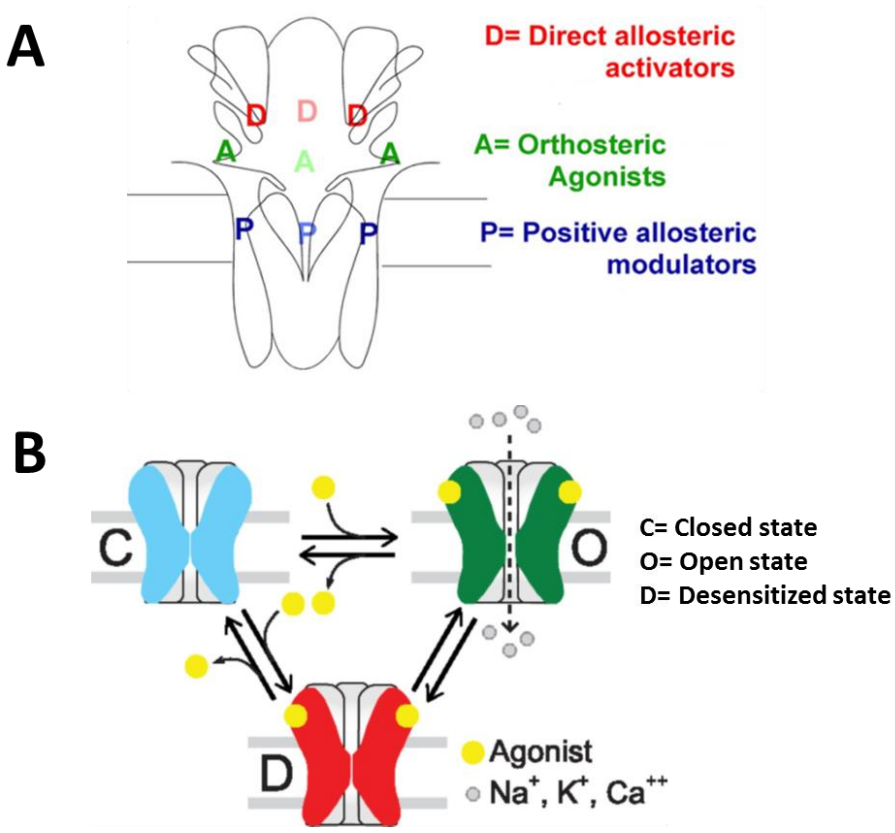
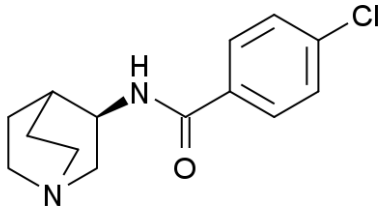
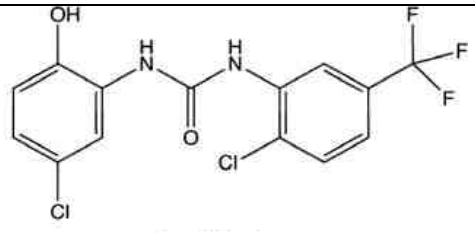
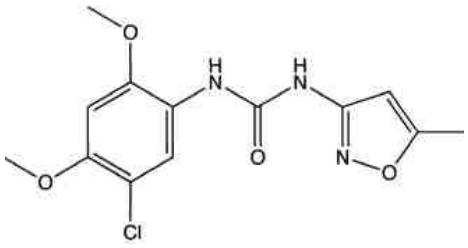
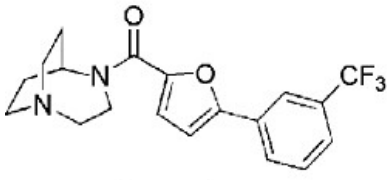


Figure 2: Schematic of proposed α7 nAChR binding sites and conformations (*Adapted from* [243,247])

A. PAMs such as NS1738 and PNU120596 are thought to bind to the PAM site (P). Ago-PAMs such as GAT107, are thought to bind two separate sites on the receptor: a PAM site (P) and a unique site for direct allosteric activation (DAA) (D). Silent Agonists (NS6740) and traditional agonists (acetylcholine or nicotine) bind to the orthosteric site (A).B. nAChRs have three general conformation states: the closed state (C), the open state (O), and the desensitized state (D). Silent agonists induce conformational changes that favor the desensitized state over the active state. Silent agonists bind to the receptor yet do not produce ion conductance of the receptor like typical orthosteric agonists.

Table 1: α7 nAChR Modulators

Name	Type of α7 nAChR Modulator	Structure, Efficacy/Potency	Selectivity for alpha 7 nAChR
PNU282987 Affinity: Ki: 27nM [248]	**Orthosteric full agonist**	 EC50 154nM [248]	> 400 times more selective for α7 than α3β4 > 100 times more selective for α7 than α4β2 nAChR [248]
NS1738 Affinity: N/A	**Type I PAM**	 ~2-3 fold increase in the maximal efficacy of ACh [232,249]	~ 8- and 26-fold selectivity for potentiation of α7 versus inhibition of α3β4 and α4β2 nAChRs respectively. [232,249]
PNU120596 Affinity: N/A	**Type II PAM**	 ~3-fold increase in the maximal efficacy of ACh [250,251]	no change in current in α4β2, α9α10 , and α3β4 nAChRs [250]
NS6740 Affinity Ki:1.1 nM [245]	**Silent Agonist**	 NS6740 efficacy: <3% of the response to ACh at both human and rat α7 nAChR[245]	> 1000 times more selective for α7 than α4β2 nAChR [245]

H. α7 nAChR Involvement in Nicotine Dependence

The α7 nAChR plays an important role in inflammation and cognition. However, there is recent evidence implicating α7 nAChRs in nicotine dependence. Polymorphisms of the CHRNA7 gene (encodes for α7 nAChR) have been linked to nicotine dependence in various human studies [252–254]. Initially, in preclinical studies null mutant mice and pharmacological studies revealed that α7 nAChRs were not necessary for nicotine reward [31,37,255] and did not play a significant role in nicotine withdrawal[93]. In nicotine CPP, a dose of nicotine (0.5mg/kg) known to produce a significant preference[78,80] was unaltered in α7 KO mice [37]. However, it was recently observed that α7 KO mice have nicotine preferences for lower doses of nicotine that do not induce a preference in their WT counterparts [35]. This observation suggested that genetic deletion of α7 nAChRs increases sensitivity to nicotine in the CPP test. Similar findings were reported with nicotine reinforcing properties. Nicotine intravenous self-administration studies either observed dose-related reduction [90,256] or no effect[255] by systemic administration of the relatively selective α7 nAChR antagonist methyllycaconitine (MLA). In contrast, selective pharmacological blockade of α7 nAChRs by the α-conotoxin ArIB in the NAc shell enhanced nicotine intake in the intravenous self-administration procedure [30]. ArIB is more than 500 times more selective for α7 nAChRs than other nAChR subtypes [257]. MLA has been shown to have off-target effects at α6*, α3*, β3* nAChRs at similar doses used to block α7 nAChRs[258]. In fact, MLA has been shown to precipitate nicotine withdrawal signs in α7 KO mice [94]. Thus, non-α7 nAChRs may be responsible for the effects of MLA in these studies and ArIB may be a more selective antagonist to probe the effect of pharmacological blockade of α7 nAChRs in nicotine reward. Similarly, the use of MLA in ICSS yielded equivocal results with reports suggesting that MLA had no effect on

nicotine-induced ICSS facilitation[90] or attenuated nicotine facilitation[259]. In the drug discrimination paradigm MLA [260] and α7 nAChR genetic deletion [261] did not alter the discriminative stimulus effect of nicotine suggesting that the α7 nAChR is not involved in this effect. Until recently, the effect of α7 nAChR activation in nicotine reward was unknown. α7 nAChR orthosteric agonists, such as PHA543613 and PNU282987, attenuated nicotine reward in the CPP test [35], and nicotine reinforcement in intravenous nicotine self-administration [30]. Similarly, α7 knock-in mice (mice heterozygous for a Leu250-to-Thr substitution in the channel domain of α7 subunit, which creates a gain-of-function mutation) had abolished nicotine preference [35]. Taken together, these studies suggest that activation of α7 nAChRs reduce the rewarding and reinforcing properties of nicotine in rodents. Interestingly, as mentioned previously, activation of α7 nAChRs and β2* nAChRs, which are required for nicotine reward [114,135]. induce dopamine release [123,136,262,263] but have divergent effects behaviorally in nicotine reward paradigms. β2* nAChR agonists substitute for nicotine in self-administration [264] drug discrimination [265] and facilitate ICSS [266]. In contrast, α7 nAChR agonists do not substitute for nicotine in drug discrimination [265] facilitate ICSS [266] or induce self-administration [30]. Collectively, this suggests that α7 nAChRs may play a modulatory role on nicotine reward in comparison to β2* nAChRs. There is a need to understand signaling pathways involved in this effect.

There is limited literature implicating α7 nAChRs in the nicotine withdrawal syndrome. Nicotine withdrawn α7 KO mice exhibit an attenuation of hyperalgesia [93,187]. There have been reports suggesting that α7 KO mice do not exhibit altered somatic signs compared to their WT counterparts[93,185,187]; however, one study observed a reduction in somatic signs in α7 KO mice [94]. The latter study may differ from the previous reports due to the different somatic signs that were

recorded. The α7 nAChR antagonist MLA precipitates a subset of nicotine withdrawal somatic signs [89,94,95] while in another study MLA had no effect [90]. This may be due to species difference. The studies that observed precipitation of somatic signs by MLA used mice while MLA had no effect in rats. α7 KO mice withdrawn from nicotine had an attenuation of anhedonia as measured by ICSS [185], but anxiety-like behavior and CPA is unaffected in α7 KO mice [93]. This suggests that different mechanisms may underlie these affective nicotine withdrawal signs. A recent study investigated the effect of α7 nAChR activation in nicotine withdrawal. The α7 full agonist ABT-107 attenuated nicotine withdrawal-induced anxiety as measured in the novelty-induced hypophagia (NIH) test [267]. There is a need of further investigation of α7 nAChR activation in nicotine withdrawal.

I. Possible Mechanisms of α7 nAChRs in Nicotine Dependence

The high Ca^{2+} permeability of the α7 nAChR results in increases of intracellular $Ca^{2+,}$ causing the opening of other channels such as voltage dependent Ca^{2+} channels[227], consequently resulting in neurotransmitter release. α7 nAChR activation can also activate Ca^{2+}-dependent signaling pathways. In preclinical cognitive studies, α7 nAChRs enhance cognition by activating extracellular receptor kinase (ERK) /mitogen-activated protein kinase (MAPK) and cyclic AMP response element-binding protein (CREB) signaling in a Ca^{2+}-dependent manner [268–270] α7 nAChR activators are undergoing clinical trials to treat cognitive disorders [271].

In addition, evidence suggests that the α7 nAChRs bind guanosine triphosphate-binding proteins (G proteins) to induce a Ca^{2+}-mediated or channel independent signaling cascades involved in dendrite plasticity [272,273]. In support of the metabotropic nature of the α7 nAChRs, the α7 nAChR silent agonist NS6740 displayed analgesic-like properties in a neuropathic pain

model[246]. Silent agonists render the receptor in a nonconductive state, thus it is plausible that the analgesic effects of the α7 nAChRs are modulated through metabotropic signaling. Indeed, α7 nAChRs on non-conducting cells such as macrophages are required for acetylcholine induced inhibition of pro-inflammatory cytokine production [274]. In addition, evidence suggests that α7 nAChRs modulate Ca^{2+}-independent signaling pathways such as the Janus kinase 2 (JAK2)/signal transducer and activator of transcription (STAT) in immune cells which may have implications in inflammation [275]. Also recently, genomic analysis has suggested that the *Chrna7* gene in mice (encodes for the α7 nAChR) regulates an insulin gene expression network in the NAc[35]. Future pharmacological and genetic investigations may clarify this possible interaction. The previously mentioned signaling cascades provide evidence that α7 nAChRs not only act as ionotropic receptors, but metabotropic properties as well.

Recently, PPARα has been shown to modulate the rewarding properties of nicotine [115]. PPARα is a transcription factor classically involved in inflammation and lipolysis [276]. Activation of PPARα reduces nicotine reward and reinforcement [34,154,277]. It has been hypothesized that α7 nAChR activation might indirectly lead to downregulation of β2* nAChRs via PPARα-induced phosphorylation of these subunits [116,278]. Since β2* nAChRs are required for nicotine reward and reinforcement [37,135], this pathway could provide an explanation of the effects of α7 nAChR activation in nicotine reward studies. Indeed, it has been shown that α7 nAChRs may fine-tune nicotine-induced DA neuron firing only after β2* nAChRs have been activated [279]. This suggests that α7 nAChRs may indirectly regulate β2* nAChRs function. Therefore, it is imperative to investigate this possible signaling pathway in nicotine dependence. Fig. 3 displays a proposed model implicating PPARα as a possible downstream mediator of α7 nAChRs activation in nicotine reward.

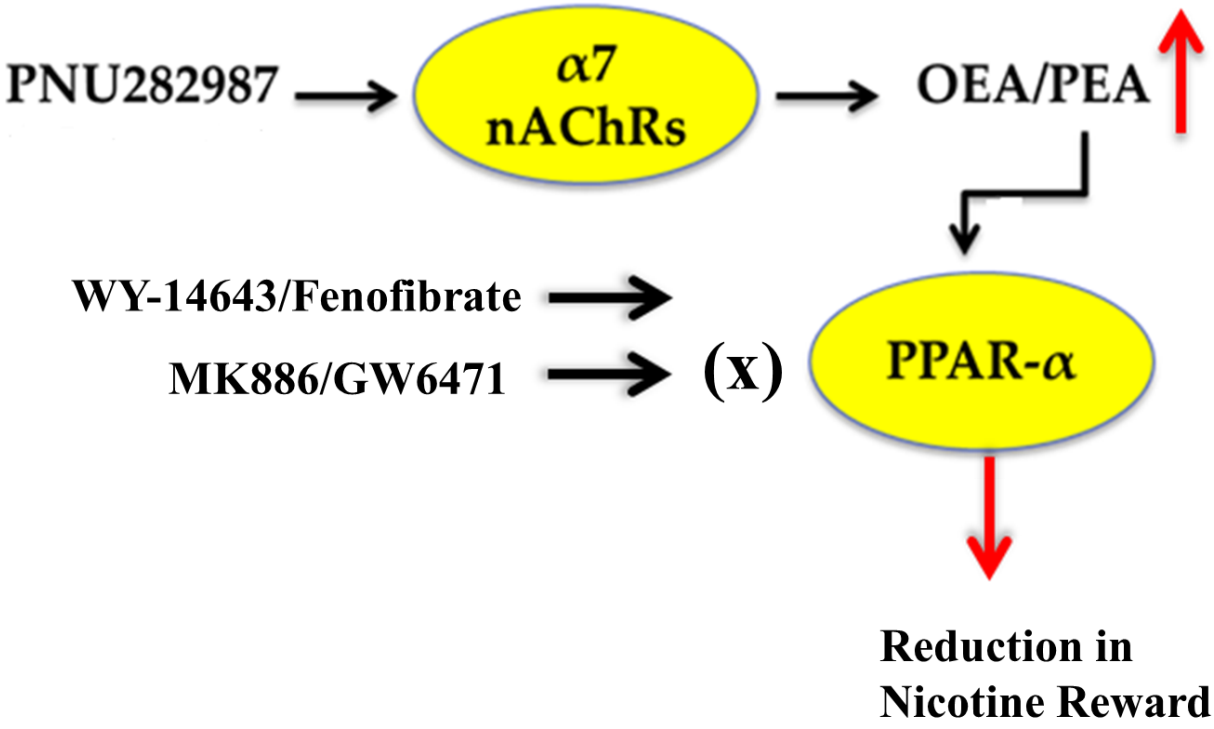


Figure 3: Schematic diagram of the proposed mechanism of PPARα and α7 nAChR interaction in nicotine reward (*Adapted from* [116])

Activation of α7 nAChRs induced by an exogenous agonist such as PNU282987 induces Ca^{2+} influx. This stimulates the synthesis of the endogenous PPARα agonists, oleoylethanolamide (OEA) and palmitoylethanolamide (PEA). These molecules then activate PPARα, which may reduce nicotine dependence. PPARα can be activated with exogenous agonists such as WY-14643 and the clinically used drug to treat high cholesterol, fenofibrate. PPARα can be blocked with antagonists such as MK886 and GW6471. This mechanism will be investigated using the mentioned pharmacological ligands in chapter 3.

J. Dissertation Aims

We hypothesize that the role of the α7 nAChR in nicotine dependence requires ion conductance and is PPARα mediated. To test this hypothesis this dissertation: 1) investigated the impact of pharmacological modulation of α7 nAChR in mouse models of nicotine dependence and 2) evaluated a possible role for PPARα as a downstream mediator of α7 nAChR in nicotine dependence. The effect of the α7 nAChR Type I PAM NS1738, Type II PAM PNU120596, and silent agonist NS6740 are unknown in nicotine reward and withdrawal assays. The utilization of these pharmacological tools will aid in the understanding of probability of channel opening (NS1738, PNU120596), desensitization (PNU120596, NS6740), and modulation of the endogenous acetylcholine/ choline tone (NS1738, PNU120596) as it relates to the α7 nAChR in nicotine dependence studies. In addition, it is unknown whether an α7 nAChR and PPARα interaction exists in nicotine dependence. There is evidence to suggest that α7 nAChR activation attenuates nicotine reward; however, the mechanism is not well understood. Recently the nuclear receptor PPARα has been shown to attenuate nicotine reward and reinforcement. Futhermore, a study indicated that α7 nAChRs may indirectly activate PPARαs. This interaction has not been investigated in nicotine reward and PPARα activation has not been studied in nicotine withdrawal thus this dissertation seeks to elucidate the role of α7 nAChR and PPARα in nicotine dependence using pharmacological interventions.

CHAPTER TWO

Modulation of the α7 Nicotinic Acetylcholine Receptor in Nicotine Dependence

A. Introduction

Even though there are many well-known health risks associated with tobacco use, tobacco dependence remains one of the leading sources of preventable death worldwide [204,280]. The current pharmacological interventions available have modest efficacy [26]; therefore, there is a need for a better understanding of the neural substrates involved in nicotine dependence to design and develop more effective smoking cessation aids. Nicotine dependence can be divided into two parts: nicotine reward and nicotine withdrawal. Both of these aspects of nicotine dependence have been investigated and the main molecular targets that have been studied are nAChRs. nAChRs are the primary target of nicotine, the addictive component in tobacco products. These receptors exist in multiple subtypes; however, the most predominate nAChRs in the mammalian brain are the homomeric α7 and heteromeric α4β2* (where *denotes the possible inclusion of additional nAChR subunits) respectively [49]. Even though activation of α7 nAChRs has been shown to induce dopamine release in the mesolimbic pathway [262,281], early behavioral studies suggested little involvement of the α7 nAChR in nicotine reward [255,260]. However, recently it has been shown that ArIB, a selective α7 nAChR antagonist, infused in the NAc shell increased nicotine intake in nicotine intravenous self-administration procedure [30]. Similarly, the genetic deletion of α7 nAChR in mice enhanced nicotine reward as measured in the CPP test [35]. In contrast, α7 knock-in mice (mice heterozygous for a Leu250-to-Thr substitution in the channel domain of α7 subunit, which creates a gain-of-function mutation) had abolished nicotine preference [35]. Furthermore, PNU282987, an α7 nAChR agonist, infused

locally into the NAc shell was found to reduce nicotine intake in intravenous self-administration in rats. This suggests that the α7 nAChR may play a modulatory role in nicotine dependence that is in contrast to β2* nAChRs, which are required for nicotine reward [37,135] (please see Ch.1 Section H for more details).

The role of the α7 nAChR in nicotine withdrawal has not been studied extensively. Nicotine withdrawal, the primary negative reinforcer that strengthens nicotine dependence, is one of the primary causes of high tobacco relapse rates [29]. In humans, it consists of somatic signs such as bradycardia, as well as non-somatic signs such as anxiety and depression [282]. The physical signs of nicotine withdrawal in rodents is measured by the observation of somatic signs, hyperalgesia and affective signs such as anxiety-like behaviors [89,283]. Few studies have been performed utilizing null mutant α7 mice in nicotine withdrawal. α7 knockout mice rendered dependent on nicotine showed a reduction in hyperalgesia [93,187],no alterations in their somatic signs [93,187] and attenuated anxiety-like behavior compared to their wild type counterparts [185]. Pharmacological blockade of the α7 receptor with MLA has been shown in some studies to precipitate a subset of nicotine withdrawal somatic signs in rats and mice [89,94,95] while in other studies MLA was ineffective at inducing nicotine withdrawal signs [90]. Recently, the α7 nAChR agonist ABT-107 was shown to reduce nicotine withdrawal-induced anxiety-like behaviors in mice [267].

There is a need for further investigation of the role of α7 nAChRs in nicotine dependence. The homomeric α7 nAChR has unique features of high calcium permeability, rapid desensitization and low probability of channel opening [42,50]. The recent development of α7 nAChR modulators such as PAMs and silent agonists may aid in understanding these characteristics in nicotine dependence paradigms. A Type I PAM such as NS1738 enhances the channel opening probability of α7 nAChRs while the Type II PAM, PNU120596 not only

increases the opening probability, but slows the desensitization rate of the receptor which results in prolonged channel opening[235,236]. The α7 nAChR silent agonist NS6740 is an orthosteric ligand that desensitizes the receptor by inducing conformational changes that favor the desensitization state over the active state [246] (please see Ch. 1 Section G for more details). To date, the impact of these α7 nAChR modulators in nicotine dependence paradigms are unknown.

Therefore, the current study investigated the physiological properties of the α7 nAChR in the nicotine CPP and nicotine withdrawal tests. The Type I PAM NS1738 and Type II PAM PNU120596 were used to evaluate the effect of channel opening probability and modulation of endogenous acetylcholine/ choline tone. The Type II PAM PNU120596 and silent agonist NS6740 were used to evaluate the role of desensitization and channel opening in nicotine dependence. The orthosteric full agonist PNU282987 was used as a reference compound. The findings of this study will advance the understanding of the α7 nAChR in nicotine dependence.

B. Materials and Methods

Animals

Drug-naive, ICR male mice (8 weeks old upon arrival; Harlan Laboratories, Indianapolis, IN) served as subjects. Mice were housed four per cage with ad libitum access to food and water on a 12-h light cycle in a humidity and temperature controlled vivarium that was approved by the Association for Assessment and Accreditation of Laboratory Animal Care. Mice received corn cob bedding and were fed Envigo Teklad mouse/rat diet 7102 (LM-485). Experiments were performed during the light cycle and were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University and followed the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Drugs

(−)-Nicotine hydrogen tartrate [(−)-1-methyl-2-(3- pyridyl) pyrrolidine (+)-bitartrate] and mecamylamine HCl (non-selective nAChR antagonist) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). PNU120596 [1-(5-Chloro-2, 4-dimethoxy-phenyl)-3-(5-methyl-isoxazol-3-yl)] and PNU282987 [N-(3R)-1 Azabicyclo [2.2.2] oct-3-yl-4-chlorobenzamide] were obtained from the National Institute on Drug Abuse (NIDA) supply program (Bethesda, MD). NS6740 (1,4-diazabicyclo[3.2.2]nonan-4-yl(5-(3-(trifluoromethyl) phenyl) furan-2-yl) methanone) was prepared as previously described (Peters et al., 2004). NS1738 was purchased from Tocris Biosciences (Minneapolis, MN). Nicotine, NS6740, mecamylamine,and PNU282987 were dissolved in physiological saline. NS1738 and PNU120596 were dissolved in a mixture of 1:1:18 [1 volume ethanol/1 volume Emulphor-620 (Rhone-Poulenc, Inc., Princeton, NJ) and 18 volumes distilled water]. Nicotine and PNU282987 were injected s.c. while all other drugs were administered intraperitoneally (i.p.). The nicotine solution pH was neutralized with sodium bicarbonate as needed. Freshly prepared solutions were given to mice at 10 ml/kg, s.c. Doses are expressed as the free base of the drug.

Nicotine conditioned place preference studies

An unbiased CPP paradigm was performed, as we previously described[284]. Briefly, the CPP apparatus consisted of three chambers in a linear arrangement (Med Associates, St Albans, VT). The CPP apparatus (MedAssociates, St. Albans, VT, ENV3013) consisted of white and black chambers (20×20×20 cm each), which differed in overall color and floor texture (white mesh or black rod). These chambers were separated by a smaller gray chamber with a smooth PVC floor.

Partitions could be removed to allow access from the gray chamber to the black and white chambers. On day 1, animals were confined to the middle chamber for a 5-min habituation and then allowed to freely move between all three chambers for 15 min. Time spent in each chamber was recorded, and these data were used to populate groups of approximately equal bias in baseline chamber preference. Twenty-minute conditioning sessions occurred twice a day (days 2–4). During conditioning sessions, mice were confined to one of the larger chambers. The saline groups received saline in one large chamber in the morning and saline in the other large chamber in the afternoon. The nicotine group received nicotine in one large chamber and saline in the other large chamber. Treatments were counterbalanced equally in order to ensure that some mice received the unconditioned stimulus in the morning while others received it in the afternoon. The nicotine-paired chamber was randomized among all groups. Sessions were 4 hrs apart and were conducted by the same investigator. On each of the conditioning days, mice were pretreated with PNU282987 (s.c.), NS1738 (i.p.) PNU120596 (i.p.), NS6740 (i.p.) or their respective vehicle 15 min prior to nicotine injection. On test day (day 5), mice were allowed access to all chambers for 15 min in a drug free state. The preference score was calculated by determining the difference between the time spent in the drug paired side during test day versus the time in drug paired side during the baseline day.

Nicotine Precipitated Withdrawal Studies

Mice were infused with 24 mg/kg/day nicotine or saline for 14 days using s.c. osmotic MPs (model 2000; Alzet Corporation, Cupertino, CA) that were implanted under isoflurane anesthesia. The concentration of nicotine was adjusted according to animal weight and mini pump flow rate. On the morning of day 15, mice were injected with vehicle, PNU120596 (3,

9 mg/kg, i.p.), PNU282987 (1, 3, 9 mg/kg, s.c.) or NS1738 (1,10 mg/kg, i.p.) 15 min before the challenge with the nonselective nAChR antagonist, mecamylamine (2 mg/kg, s.c.), that was administered 5 min after vehicle or drugs. Withdrawal assessment was performed 10 min later as described in [93]. Affective (anxiety-like behavior) and physical (somatic signs, hyperalgesia) nicotine withdrawal signs were evaluated in this paradigm. Mice were first evaluated for 5 min in the plus maze test for anxiety-related behavior. Time spent on the open arms of the plus maze was assessed as a measure of anxiety-related response. The number of arm crosses between the open and closed arms was also counted as a measure of locomotor activity. The plus maze assessment was immediately followed by a 20-min observation of somatic signs measured as paw and body tremors, head shakes, backing, jumps, curls, and ptosis. Mice were placed in clear activity cages without bedding for the observation period. The total number of somatic signs was tallied for each mouse and the average number of somatic signs during the observation period was plotted for each test group. Hyperalgesia was evaluated using the hot plate test immediately following the somatic sign observation period. Mice were placed into a 10-cm wide glass cylinder on a hot plate (Thermojust Apparatus, Richmond, VA) maintained at 52°C. The latency to reaction time (jumping or paw licking) was recorded. The specific testing sequence was chosen based on our prior studies showing that this order of testing reduced within-group variability and produced the most consistent results [93]. All studies were performed by an observer blinded to experimental treatment.

Statistical analysis

Data were analyzed using the GraphPad software version 6.0 (GraphPad Software, Inc., La Jolla, CA) and expressed as the mean ± S.E.M. A one-way analysis of variance (ANOVA) in

conjunction with Holm-Šídák comparison tests were conducted to determine significant effects of drug treatments vs controls. Comparisons were considered statistically significant when $p < 0.05$.

C. Results

Nicotine CPP attenuated by α7 nAChR full orthosteric agonist PNU282987

Mice were conditioned with either saline or nicotine (0.5 mg/kg) for 3 days in the CPP paradigm. A robust CPP was observed in nicotine – conditioned mice pre-treated with vehicle [$F(4, 29) = 14.05$, $p<0.0001$]. PNU282987 reduced nicotine reward. Post hoc analysis revealed that pretreatment with a lower dose of PNU282987 (1 mg/kg) did not significantly alter nicotine CPP ($p>0.05$), but a higher dose of the agonist (9mg/kg) did ($p<0.05$) (Fig. 4). PNU282987 at the dose of 9 mg/kg did not produce a preference or aversion in saline treated-mice. PNU282987 was administered within the range of doses used for other behavior studies [265,285].

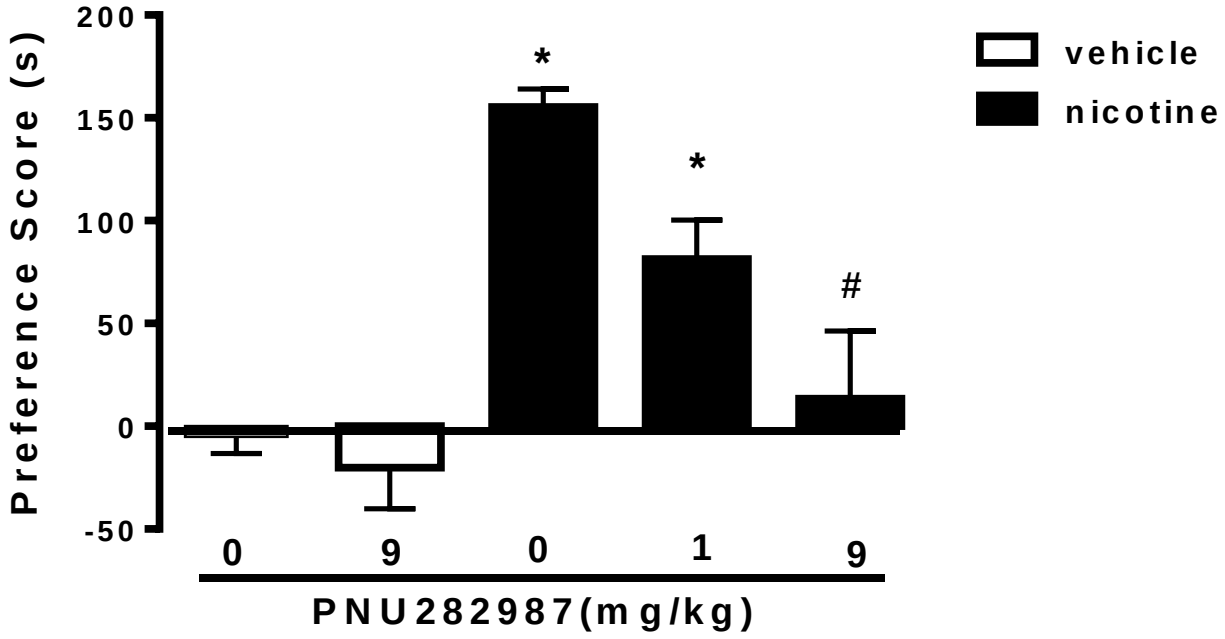


Figure 4: α7 nAChR Full Orthosteric Agonist PNU282987 Blocks Nicotine CPP

Mice underwent 3 days of conditioning with s.c. saline or nicotine (0.5mg/kg). Nicotine produced a robust CPP in mice pre-treated with vehicle. The α7 full orthosteric agonist PNU282987 (1 and 9 mg/kg; s.c.) attenuated nicotine reward as measured by the CPP. * Denotes $p<0.05$ from vehicle-vehicle. # Denotes $p<0.05$ from nicotine control. Each point represents the mean ± SEM of n=9-10 mice per group.

α7 nAChR Type I PAM NS1738 had no effect on Nicotine CPP

CPP conditioning with either saline or nicotine (0.5 mg/kg) was performed for 3 days. CPP was observed in nicotine–conditioned mice pre-treated with vehicle [$F(4, 32) = 6.434$, $p = 0.0006$] NS1738 did not reduce nicotine reward at either dose tested (1 and 10mg/kg) ($p>0.05$) (Fig. 5). NS1738 at the dose of 10 mg/kg did not produce a preference or aversion in saline treated-mice. NS1738 was used at doses previously described [240,286].

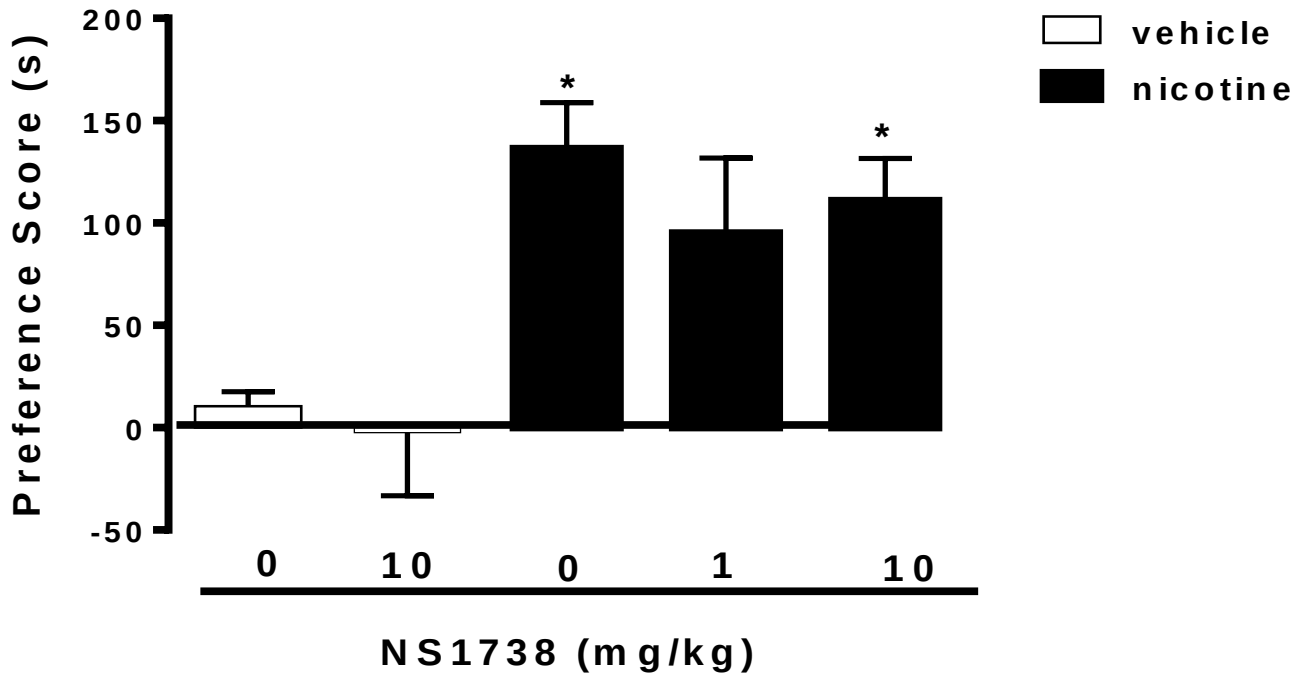


Figure 5: α7 nAChR Type I PAM NS1738 Did Not Block Nicotine CPP

Mice underwent 3 days of conditioning with either s.c. saline or nicotine (0.5mg/kg). Nicotine produced a robust CPP in mice pre-treated with vehicle. The α7 Type I PAM NS1738 (1 and 10 mg/kg; i.p.) did not alter nicotine reward as measured by the CPP test at both doses tested. * Denotes $p<0.05$ from vehicle-vehicle. Each point represents the mean ± SEM of n=6-9 mice per group.

α7 nAChR Type II PAM PNU120596 reduced Nicotine CPP

CPP conditioning with either saline or nicotine (0.5 mg/kg) was performed for 3 days. CPP was observed in nicotine–conditioned mice pre-treated with vehicle reward [$F(4, 42) = 7.864$, $p < 0.0001$]. PNU120596 significantly reduced nicotine reward. PNU120596 attenuated nicotine CPP at both doses tested (1 and 9 mg/kg) ($p<0.05$) (Fig. 6). PNU120596 at the dose of 9 mg/kg did not produce a preference or aversion in saline treated-mice. PNU120596 was used at similar doses previously described [240,241].

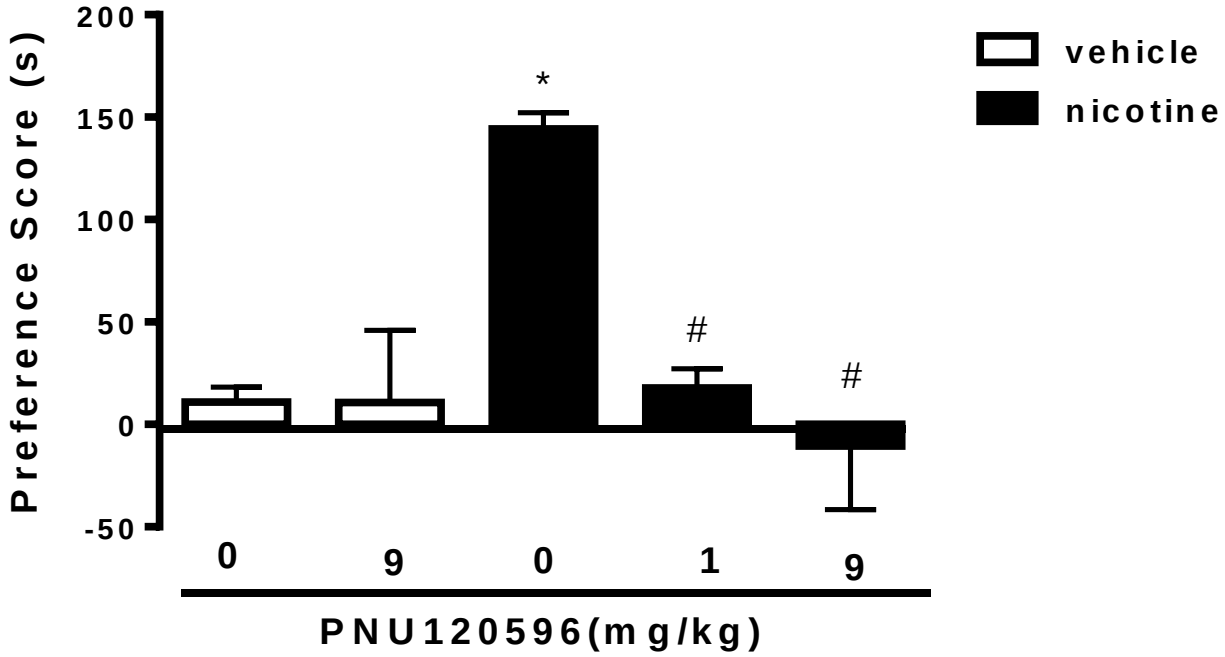


Figure 6: Attenuation of the Development of Nicotine CPP by α7 nAChR Type II PAM PNU120596.

Mice underwent 3 days of conditioning with either saline or nicotine (0.5mg/kg;s.c.). Nicotine produced a significant CPP in mice pre-treated with vehicle. The α7 Type II PAM PNU120596 (1 and 9 mg/kg; i.p.) reduced nicotine reward as measured by the CPP test at both doses tested. * Denotes $p<0.05$ from vehicle-vehicle. # denotes $p<0.05$ from nicotine control. Each point represents the mean ± SEM of n=9-10 mice per group.

α7 nAChR Silent Agonist NS6740 Did Not Attenuate Nicotine CPP

CPP conditioning with either saline or nicotine (0.5 mg/kg) was performed for 3 days. CPP was observed in nicotine – conditioned mice pre-treated with vehicle [$F(4, 36) = 6.186$ $p=0.0007$]. NS6740 had no effect on nicotine reward at both doses tested (1 and 3 mg/kg) (Fig. 7). NS6740 at the dose of 3 mg/kg did not produce a preference or aversion in saline treated-mice. NS6740 was used at doses previously described [246].

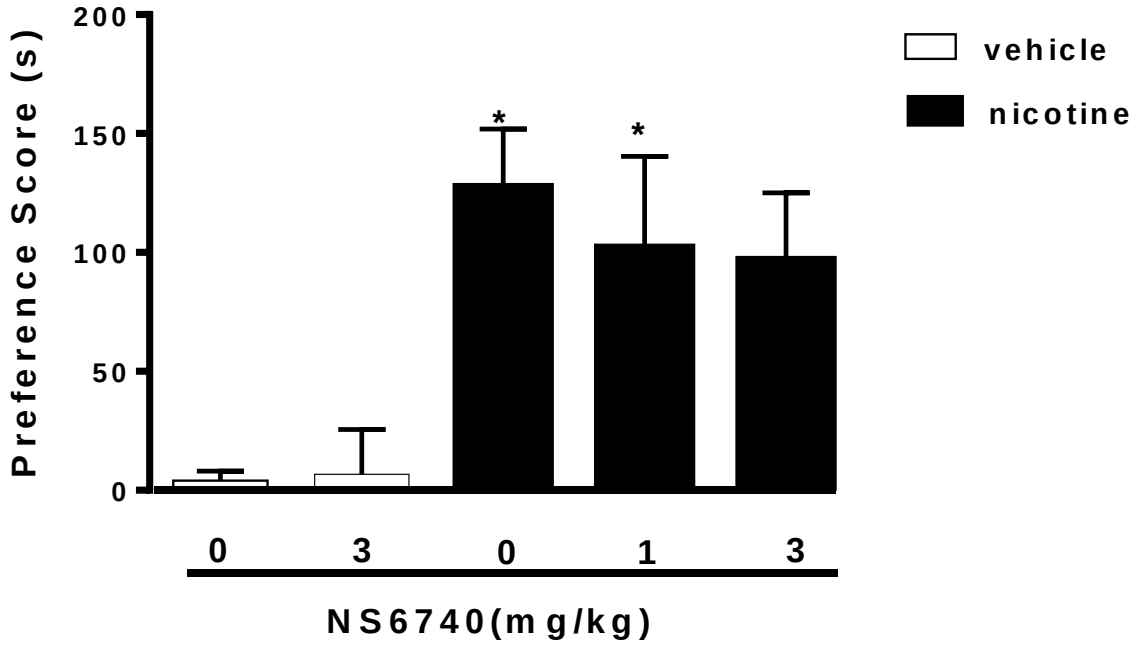


Figure 7: No Effect of α7 nAChR Silent Agonist NS6740 on the Development of Nicotine CPP.

Mice underwent 3 days of conditioning with either s.c. saline or nicotine (0.5mg/kg).Nicotine produced a robust CPP in mice pre-treated with vehicle. The α7 silent agonist NS6740 (1 and 3 mg/kg; i.p.) did not reduce nicotine reward as measured by the CPP test at both doses tested. * Denotes $p<0.05$ from vehicle-vehicle. Each point represents the mean ± SEM of n=7-10 mice per group.

α7 nAChR full orthosteric agonist PNU282987 attenuates somatic and affective nicotine withdrawal signs

The physical (somatic signs and hyperalgesia) and affective (anxiety-related behavior) signs of nicotine withdrawal were measured in mice following pretreatment with either PNU282987 or vehicle 15 min prior to mecamylamine administration on day 15. Nicotine withdrawn mice had a significantly increased anxiety-related behavior in the plus maze [$F(5, 32) = 11.21$, $p < 0.0001$] (Fig. 8A), increased expression of somatic withdrawal signs [$F(5, 32) = 24.48$, $p < 0.0001$] (Fig. 8B), and decreased response latencies in the hot-plate test [$F(5, 32) = 17.89$, $p < 0.0001$] (Fig. 8C). Mice implanted with saline MPs and received vehicle expressed no withdrawal signs. PNU282987 attenuated nicotine withdrawal signs in a dose related manner. Pretreatment with PNU282987 exhibited a trend of reducing anxiety-like behavior (time in open arms in the plus-maze test) and reached significance at 9mg/kg (s.c.) ($p<0.05$) (Fig. 8A). In addition, pretreatment with PNU282987 decreased nicotine somatic withdrawal signs and was statistically significant at doses 3 and 9 mg/kg ($p<0.05$) (Fig. 8B). However, as the post hoc analysis showed, pretreatment with PNU282987 was ineffective at attenuating the expression of hyperalgesia (hot-plate latency) at all doses tested ($p<0.05$) (Fig. 8C). The highest dose of PNU282987 tested (9 mg/kg) did not significantly affect behavioral responses in saline-infused mice in any withdrawal test.

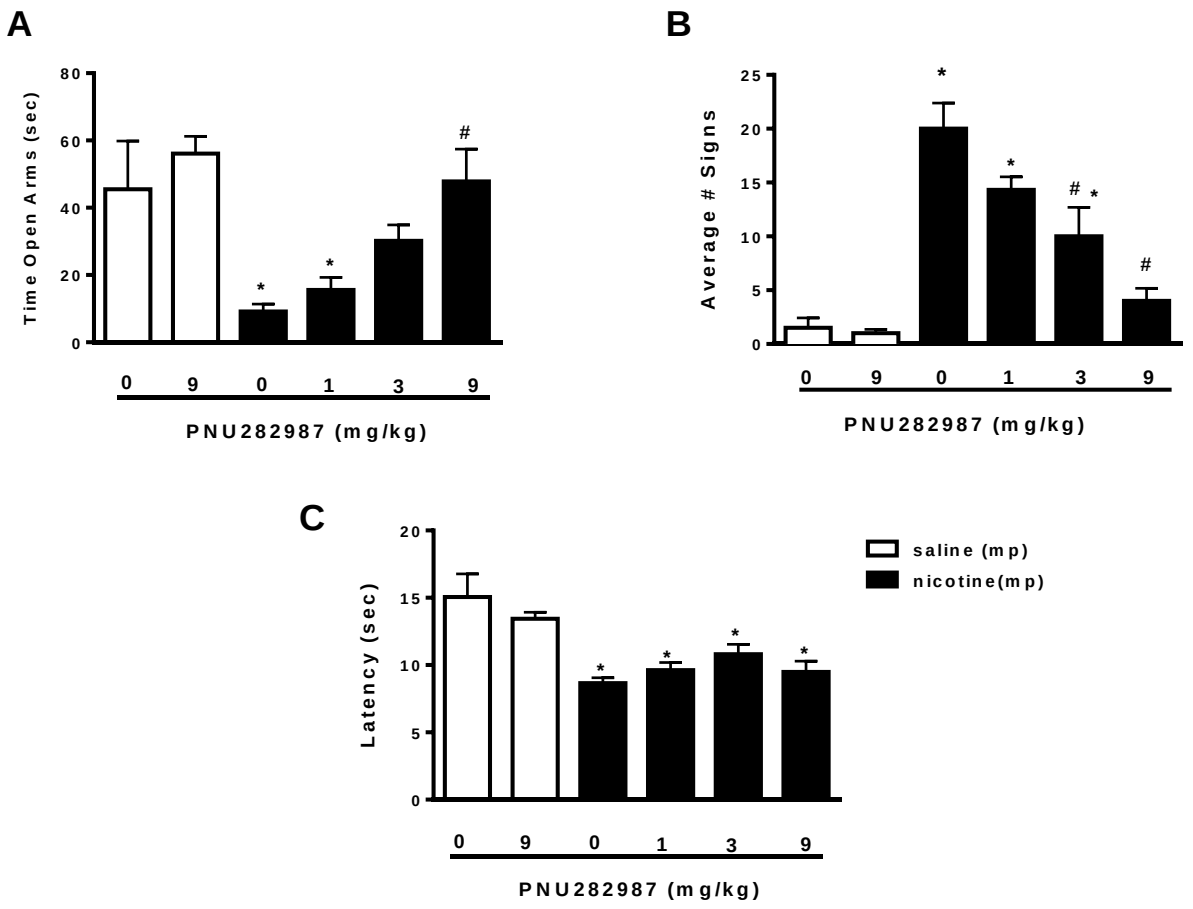


Figure 8: Effects of Full α7 Orthosteric Agonist PNU282987 on Physical and Affective Signs of Precipitated Nicotine Withdrawal

Mice were chronically infused with saline or nicotine (24 mg/kg/day) for 14 days. On day 15 mice received s.c. injection of PNU282987 (1, 3 and 9 mg/kg) or vehicle. Mice then were administered mecamylamine (2mg/kg; s.c.) 10 min prior to behavioral assessment of **A)** anxiety-like behaviors (Time spent in the open arm), **B)** somatic signs, and **C)** hyperalgesia (hot plate latency).Nicotine induced withdrawal symptoms: increased anxiety related behavior and somatic signs, but decreased hot plate latency. Compared to vehicle, pretreatment with PNU282987**: A)** attenuated the anxiety-like behavior at 9mg/kg; **B)** reduced somatic signs at 3 and 9mg/kg; and **C)** and no effect on hot plate latency in nicotine withdrawn mice. Each point represents the mean ± S.E.M. of n=6–8 mice per group. * Denotes $p < 0.05$ vs. Saline MP group, # Denotes $p < 0.05$ vs. Nicotine MP group.

α7 nAChR Orthosteric Full Agonist PNU282987 Did Not Alter Arm Crosses in the Elevated Plus Maze.

To examine whether or not the results observed in the elevated plus maze test were possibly confounded by alterations in locomotor activity induced by PNU282987 administration, the number of arm crosses were recorded. As shown in Table 2 PNU282987 had no effect on the number of arm crosses in the plus maze [$F(5, 32) = 0.7950$, $p=0.5613$].

Table 2: PNU282987 does not have an effect on the average number of arm crosses in the elevated plus maze test

Mice undergoing nicotine withdrawal received PNU282987 (1, 3 and 9 mg/kg; s.c.) or vehicle. The average number of arm crosses were recorded in the plus maze test. The numbers are presented as the total number of arm crosses ± SEM (*n*=8).

Treatment	**Average number of arm crosses ±SEM**
Saline MP-vehicle	**6.8± 0.6**
Saline MP- PNU282987 (9)	**7.2±0.7**
Nicotine MP- vehicle	**7.5±0.9**
Nicotine MP-PNU282987 (1)	**6.2±0.7**
Nicotine MP-PNU282987 (3)	**6.5 ± 0.6**
Nicotine MP-PNU282987 (9)	**5.8±0.5**

Somatic nicotine withdrawal signs are attenuated by α7 nAChR Type I PAM NS1738

Physical and affective signs of nicotine withdrawal were measured in mice following pretreatment with either NS1738 or vehicle 15 min prior to mecamylamine administration on day 15. Nicotine withdrawn mice had a significantly increased anxiety-related behavior in the plus maze [$F (4, 35) = 21.86$, $p<0.0001$] (Fig. 9A), increased expression of somatic withdrawal signs [$F (4, 35) = 37.32$, $p<0.0001$] (Fig. 9B), and decreased response latencies in the hot-plate test[$F (4, 35) = 5.208$, $p=0.0021$] (Fig. 9C). Pretreatment with NS1738 had no effect on the expression of anxiety-related behaviors (time in open arms in the plus-maze test) ($p>0.05$) (Fig. 9A). However, NS1738 reduced nicotine somatic withdrawal signs at 10mg/kg ($p<0.05$) (Fig. 9B). Pretreatment with NS1738 exhibited a trend of reversing hot plate latencies (measure of hyperalgesia) but it did not reach significance at any of the doses tested($p>0.05$) (Fig. 9C). The highest dose of NS1738 (10 mg/kg) did not significantly affect behavioral responses in saline-infused mice in any withdrawal test.

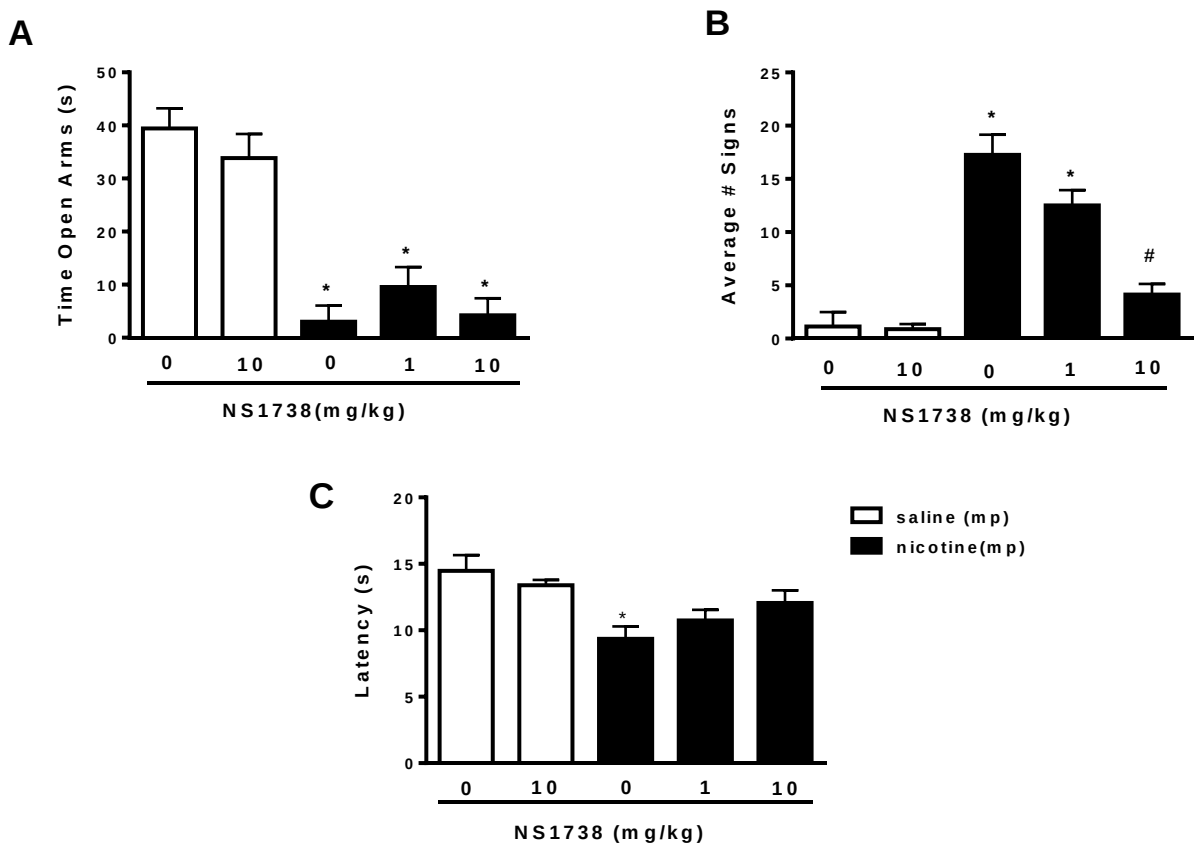


Figure 9: Effects of α7 Type I PAM NS1738 on Physical and Affective Signs of Precipitated Nicotine Withdrawal

Mice were infused with saline or nicotine (24 mg/kg/day) for 14 days. On day 15 mice received s.c. injection of NS1738 (1 and 10 mg/kg) or vehicle. Mice then were administered mecamylamine (2mg/kg; s.c.) 10 min prior to behavioral assessment of **A**) anxiety-like behaviors (Time spent in the open arm), **B**) somatic signs, and **C**) hyperalgesia (hot plate latency). Nicotine induced withdrawal symptoms: increased anxiety related behavior and somatic signs, but decreased hot plate latency. Compared to vehicle, pretreatment with NS1738: **A**) did not attenuate the anxiety-like behavior at any dose tested; **B)** reduced somatic signs at 10mg/kg; and **C**) and had no effect on hot plate latency in nicotine withdrawn mice. Each point represents the mean ± S.E.M. of n=6–8 mice per group. * Denotes $p < 0.05$ vs. Saline MP group, # Denotes $p < 0.05$ vs. Nicotine MP group.

α7 nAChR Type I PAM NS1738 Did Not Alter Arm Crosses in the Elevated Plus Maze.

To examine whether or not the results observed in the elevated plus maze test was possibly confounded by alterations in locomotor activity induced by NS1738 administration, the number of arm crosses were recorded. As shown in Table 3 NS1738 had no effect on the number of arm crosses in the plus maze [$F(4, 35) = 0.7950$, $p=0.9962$].

Table 3: NS1738 does not have an effect on the average number of arm crosses in the elevated plus maze test

Mice undergoing nicotine withdrawal received NS1738 (1 and 10 mg/kg; i.p.) or vehicle. The average number of arm crosses were recorded in the plus maze test. The numbers are presented as the total number of arm crosses ± SEM (n=8).

Treatment	**Average number of arm crosses ±SEM**
Saline MP-vehicle	**9.9± 1.8**
Saline MP- NS1738 (10)	**9.6±1.4**
Nicotine MP- vehicle	**10.1±1.4**
Nicotine MP-NS1738 (1)	**9.4±1.7**
Nicotine MP-NS1738 (10)	**9.4 ± 1.4**

Nicotine withdrawal-induced hyperalgesia attenuated by α7 nAChR Type II PAM PNU120596

Physical and affective signs of nicotine withdrawal were measured in mice following pretreatment with either PNU120596 or vehicle 15 min prior to mecamylamine administration on day 15. Nicotine withdrawn mice had a significantly increased anxiety-related behavior in the plus maze [$F(4, 29) = 3.730$, $p = 0.0144$](Fig.10A), increased expression of somatic withdrawal signs [$F(4, 30) = 19.92$, $p<0.0001$] (Fig. 10B), and decreased response latencies in the hot-plate test [$F(4, 30) = 6.808$, $p = 0.0005$] (Fig. 10C). Pretreatment with PNU120596 had a tendency to decrease the expression of anxiety-related behaviors (time in open arms in the plus-maze test), however neither dose used altered anxiety-like behaviors significantly ($p>0.05$) (Fig. 10A). In addition, PNU120596 at all doses used was ineffective at reducing nicotine somatic withdrawal signs ($p>0.05$) (Fig. 10B). However, pretreatment with PNU120596 exhibited a trend of reversing hot plate latencies (measure of hyperalgesia) and significantly increased hot plate latencies at 9mg/kg ($p<0.05$) (Fig. 10C). The highest dose of PNU120596 (9 mg/kg) did not significantly affect behavioral responses in saline-infused mice in any withdrawal test.

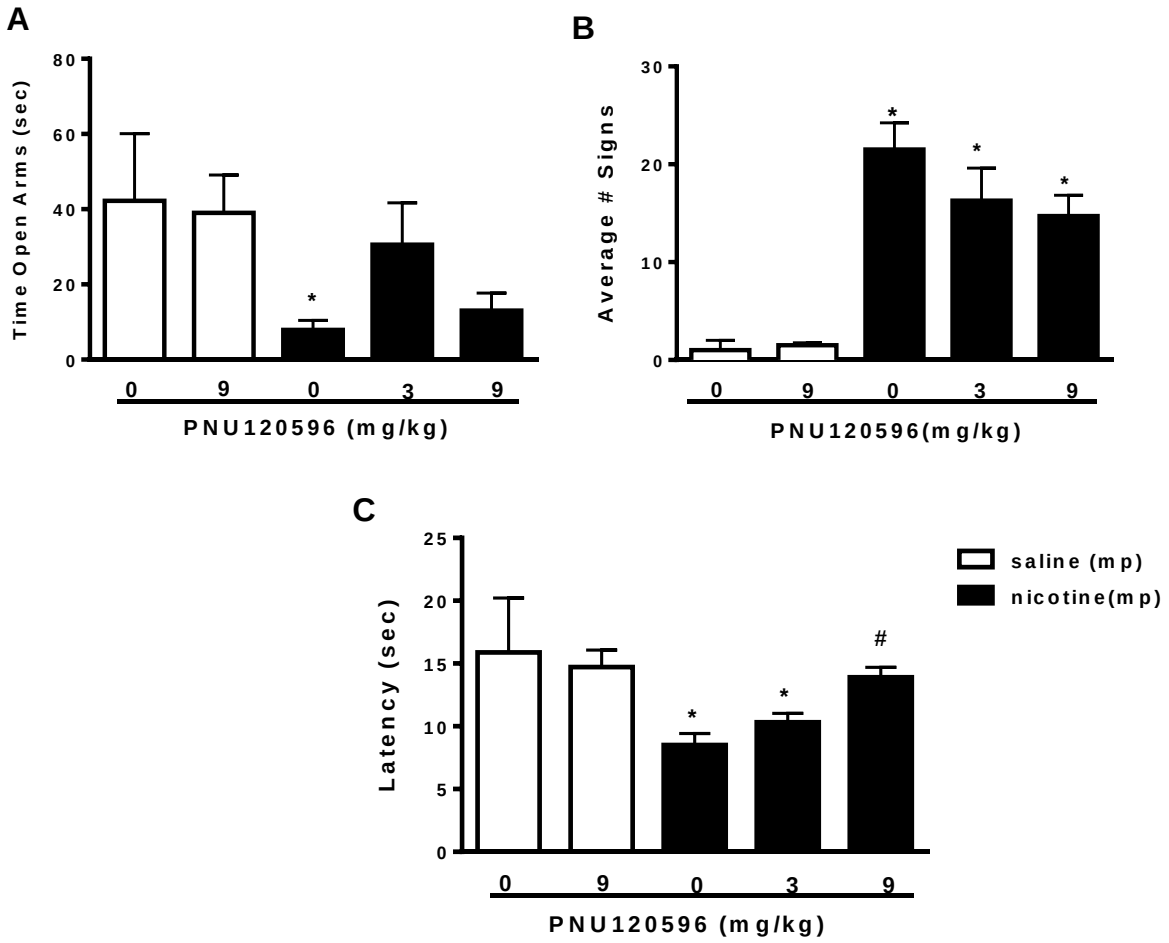


Figure 10: Effects of Type II PAM PNU120596 on Physical and Affective Signs of Precipitated Nicotine Withdrawal

Mice were chronically infused with saline or nicotine (24 mg/kg/day) for 14 days. On day 15 mice received i.p. injection of PNU120596 (3 and 9 mg/kg) or vehicle. Mice then were administered mecamylamine (2mg/kg; s.c.) 10 min prior to behavioral assessment of **A**) anxiety-like behaviors (Time spent in the open arm), **B**) somatic signs, and **C**) hyperalgesia (hot plate latency).Nicotine induced withdrawal symptoms: increased anxiety related behavior and somatic signs, but decreased hot plate latency. Compared to vehicle, pretreatment with PNU120596: **A**) had no effect anxiety-like behavior; **B**) had no effect on somatic signs; and **C**) but significantly increased hot plate latency at 9mg/kg in nicotine withdrawn mice. Each point represents the mean ± S.E.M. of n=6–8 mice per group. * Denotes $p < 0.05$ vs. Saline MP group, # Denotes $p < 0.05$ vs. Nicotine MP group.

α7 nAChR Type II PAM PNU120596 Did Not Alter Arm Crosses in the Elevated Plus Maze.

To examine whether or not the results observed in the elevated plus maze test was possibly confounded by alterations in locomotor activity induced by PNU120596 administration, the number of arm crosses were recorded. As shown in Table 4 PNU120596 had no effect on the number of arm crosses in the plus maze [$F(5, 32) = 0.5965$, $p=0.6682$].

Table 4: PNU120596 does not have an effect on the average number of arm crosses in the elevated plus maze test

Mice undergoing nicotine withdrawal received PNU120596 (3 and 9 mg/kg; i.p.) or vehicle. The average number of arm crosses were recorded in the plus maze test. The numbers are presented as the total number of arm crosses ± SEM (*n*=8).

Treatment	**Average number of arm crosses ±SEM**
Saline MP-vehicle	**7.5± 0.9**
Saline MP- PNU120596 (9)	**6.9±0.8**
Nicotine MP- vehicle	**7.8±0.8**
Nicotine MP-PNU120596 (3)	**6.5±0.4**
Nicotine MP-PNU120596 (9)	**7.9 ± 0.9**

D. Discussion

The results of this study produced interesting findings about the impact of α7 nAChR modulation and conformations on nicotine reward and withdrawal in mice. The α7 full orthosteric agonist PNU282987 and the Type II α7 nAChR PAM PNU120596 reduced nicotine CPP (Fig. 4 and 6) while the silent agonist NS6740 and Type I PAM NS1738 had no effect (Fig. 5 and 7). In nicotine withdrawal, PNU282987, NS1738, and PNU120596 attenuated different signs of the withdrawal syndrome (Fig.8, 9 and 10). To our knowledge, this is the first report of α7 nAChR PAMs and a silent agonist used in preclinical nicotine dependence tests.

In the presence of an orthosteric full agonist, the α7 nAChR has a low probability of opening, is permeable to calcium and rapidly desensitizes[42,50]. These intrinsic factors may limit the usefulness of α7 nAChR ligands; therefore, PAMs were developed as pharmacological tools to circumvent the intrinsic limitations of the α7 nAChR. The probability of an α7 nAChR being open is less than one in a million [51], thus the Type I PAM NS1738 and Type II PAM PNU120596 , which increase the probability of channel opening, were used to evaluate the role of this α7 nAChR feature in nicotine CPP. In comparison to the traditional orthosteric agonist PNU282987, which attenuated nicotine CPP at 9mg/kg, PNU120596 reduced nicotine CPP at both doses used (1 and 9mg/kg). PNU120596 may be more potent than PNU282987 in the nicotine CPP test. Utilizing multiple doses of these compounds will further characterize this observation. NS1738 had no effect on nicotine at both doses tested (1 and 10mg/kg). This suggests that the Type I PAM NS1738 does not reveal the anti-reward endogenous tone mediated by α7 nAChRs with an increased probability of channel opening. The divergent effects of the Type I and Type II PAM may be the result of PNU120596's ability to decrease the rate of desensitization. PNU120596 not only increases the chance of ion conductance but also allows

the channel to remain in the open state for a longer duration, which also results in an increase of possible ion conductance. Attenuating the desensitization rate of the endogenous tone by PNU120596 was sufficient to induce an effect in nicotine CPP. Similar findings with NS1738 and PNU120596 were shown in a mouse model of tonic pain. The Type II PAM PNU120596, but not the Type I PAM NS1738, reduced pain-related behaviors in the early and late phase of the formalin test [240]. Nicotine CPP is a CNS-mediated effect [36,139], thus, the lack of effect of NS1738 may be due to poor blood brain barrier penetrability. However, systemic administration of NS1738 at similar doses used in our study produced brain concentrations [240] that were shown to enhance the channel opening of acetylcholine *in vitro* [249]. In addition, it has been previously reported that NS1738 treated mice do not exhibit any motor impairments or alterations in their locomotor activity [286]. This current study also confirms the lack of effect of NS1738 on locomotor activity as indicated by the number of crossovers in the elevated plus maze (see Table 3). Furthermore, there are thought to be at least two types of desensitization states for the α7 nAChR: Type II modulator sensitive and Type II modulator insensitive [42]. NS1738 and PNU120596 may induce different desensitization states, which may be responsible for the divergent results. However, differentiation and effects of these two type of desensitization states are unknown in vivo. Our results with the silent agonist NS6740 (1 and 3mg/kg), which induces the receptor into a desensitized state with the absence of an open state, did not alter nicotine CPP. Higher doses of NS6740 were not use due to aversion it caused on its own. NS6740 is effective at reducing chronic pain and inflammation in mice [246]; however, it lacked efficacy in cognitive assays [245]. This suggests that centrally mediated effects of nicotine may require ion conductance of α7 nAChRs.

In our nicotine withdrawal experiments the orthosteric agonist PNU282987 attenuated anxiety-like behaviors (Fig.8); however, the α7 nAChR PAMs NS1738 and PNU120596 had no effect on anxiety-like behavior as observed in the elevated plus maze (Fig. 9 and 10). This suggests that low probability of channel opening and rapid desensitization are needed for this effect. Our results are in agreement with a recent study of α7 nAChR activation with the α7 orthosteric agonist ABT-107 which was shown to also attenuate anxiety-like behaviors in the NIH test [267]. However, in another study α7 nAChR KO mice that received 36mg/kg/day of nicotine for 14 days and underwent precipitated withdrawal, did not show alterations in anxiety-related behaviors or CPA [93]. It has been previously shown in reward studies that α7 KO mice may have an increased sensitivity to nicotine at lower doses [35] and this sensitivity is undetectable at higher typical rewarding doses [37]. Thus, the lack of alteration of anxiety-like behavior in the α7 KO mice may be due to the high dose of nicotine given to mask an effect. Indeed, a lower dose such as 24mg/kg/day of nicotine for 14 days has also been shown to produce reliable nicotine withdrawals [89,182] and this dose is used in the current study.

The orthosteric full agonist PNU282987 and the Type I PAM NS1738 both attenuated somatic signs, but the Type II PAM PNU120596 had no effect on somatic signs. This may suggest that rapid desensitization is necessary for the attenuation of somatic signs by α7 nAChRs. It has been shown that α7 nAChR KO mice undergoing nicotine withdrawal have a reduction in somatic signs [94],implicating the importance of α7 nAChR blockade or desensitization in nicotine dependence. However, another study from our lab that measured the same somatic signs did not see a reduction in somatic signs observed in α7 KO mice compared to their WT littermates [93]. This discrepancy may be contributed to the different somatic signs observed in the studies. The latter study observed somatic signs such as paw tremors, body tremors, and backing while the

former study tallied signs such as grooming, scratching, and chewing. Therefore, the somatic sign results should be interpreted with caution.

PNU120596 was the only α7 ligand that reduced nicotine withdrawal-induced hyperalgesia in the hot plate test. It is unclear of the reason for this reduction of hyperalgesia by PNU120596 and the lack of effect of PNU282987. Previous studies implicate the α7 nAChR in the reduction of hyperalgesia evidenced in nicotine withdrawn α7 KO mice [93,187]. In contrast, the α7 nAChR antagonist MLA has been shown to precipitate hyperalgesia [89]. This effect may be to the antagonism of MLA at off-target effects such as α6*, α3*, β3* nAChRs [258]. To further evaluate the role of α7 nAChR desensitization in nicotine withdrawal, the silent agonist NS6740 should be utilized.

Taken together, our results suggests that desensitization/ion conductance and channel opening of the α7 nAChR play important roles in nicotine dependence behaviors in mice. In addition, the utilization of PAMs in this study suggests that endogenous acetylcholine/ choline tone is sufficient to attenuate some aspects of nicotine withdrawal. These findings highlight a beneficial effect of using α7 PAMs instead of α7 orthosteric agonists. PAMs may provide less overstimulation to the endogenous cholinergic system because activation will only occur in the presence of acetylcholine release. In addition, PAMs also provide better selectivity for α7 nAChRs. They interact with an allosteric site of the receptor and α7 nAChRs and serotonin 5-HT_3 receptors have a high homology of their ligand binding domain [237]. The silent agonist NS6740 used in this study aided to understand the role of desensitization and ion conductance of the α7 nAChR. PAMs and silent agonists may serve as useful tools to understand the effect of α7 nAChR modulation in nicotine dependence.

CHAPTER THREE

In vivo Interactions between α7 Nicotinic Acetylcholine Receptor and Nuclear Peroxisome Proliferator-Activated Receptor-α: Implication for Nicotine Dependence

The published article below with the addition of two supplemental figures (Fig. 14 and Fig. 18) was used for chapter three.

Jackson A, Bagdas D, Muldoon PP, Lichtman AH, Carroll FI, Greenwald M, Miles MF, Damaj MI. In vivo interactions between α7 nicotinic acetylcholine receptor and nuclear peroxisome proliferator-activated receptor-α: Implication for nicotine dependence. Neuropharmacology. 2017 Mar 7;118:38-45.

A. Introduction

The homomeric α7 nicotinic acetylcholine receptor (nAChR) has been shown to play a role in cognition, inflammation, immunity and neuroprotection [247]. Recent findings suggest this low affinity α7 nAChR modulates nicotine reward and reinforcement in rodents [30,35]. The α7 nAChR selective agonist PNU282987 infused locally into the nucleus accumbens (NAc) shell reduced intravenous self-administered nicotine in rats. In contrast, ArIB, an α7 selective nAChR antagonist, infused in the NAc increased nicotine intake [30]. Similarly, the genetic deletion of α7 nAChRs in mice enhances nicotine reward as measured in the CPP test, whereas α7 knock-in (producing mice heterozygous for a Leu250-to-Thr substitution in the channel domain of α7 subunit, which creates a gain-of-function mutation) abolishes nicotine preference. In addition, the selective α7 agonist PHA-543613 blocked the development of nicotine CPP in mice [35]. Attenuation of nicotine reward and reinforcement by α7 nAChR agonists seems to be associated with a decreased nicotine-induced dopaminergic transmission in the brain, as PNU282987 blocks

nicotine-induced increased firing activity of the ventral tegmental area (VTA) dopamine neurons in rats [116].

This important effect of α7 nAChR modulation of nicotine reward has prompted studies of the underlying mechanism. It has been suggested that α7 nAChR activation regulates VTA dopaminergic cells via the PPARα in the rat. The α7 nAChR agonist PNU282987 induced synthesis of two fatty acid PPARα endogenous ligands, OEA and PEA, that in turn activate PPARα and phosphorylate β2-containing nAChRs on dopamine neurons via a tyrosine kinase pathway [116]. These findings suggest a pathway by which α7 nAChR pharmacological stimulation indirectly inactivates β2-containing nAChRs via PPARα receptors. However, the above-noted study did not directly investigate this mechanism using a nicotine reward paradigm which is imperative because β2-containing nAChRs are required for nicotine reward [37,135].

PPARα is a nuclear ligand-activated transcription factor that when activated, enhances transcription of various genes involved in modulating many peripheral physiological responses such as inflammation and lipolysis [276]. Importantly, PPARαs, which are located in brain regions associated with reward [287–289], have been shown to modulate the rewarding properties of abused substances such as alcohol and nicotine [115,290]. Acute administration of PPARα agonists attenuates nicotine [34,154,277] and alcohol reinforcement [290], alcohol intake [291,292] and nicotine-induced dopamine firing in rodents [115]. For example clofibrate, a lipid lowering agent and PPARα agonist [293], was shown in rats to block acquisition of nicotine seeking, decrease nicotine intravenous self-administration and block nicotine-induced dopamine release into the NAc shell [34]. Therefore, we hypothesize that PPARα may serve as a downstream mediator of α7 nAChR activation in nicotine reward. To test this hypothesis the present study investigated the interaction of the α7 nAChR and PPARα in a preclinical mouse model of reward (nicotine CPP).

Furthermore, we examined PPARα activation in nicotine CPP and nicotine withdrawal, a behavioral outcome not measured before in preclinical studies with PPARα activators. We compared effects of the selective and potent PPARα agonist WY-14643 [294,295] with a commonly used lipid lowering fibrate medication that activates PPARα, fenofibrate [296]. Results from these experiments may provide insight into the roles of α7 nAChR and PPARα in nicotine dependence.

B. Materials and Methods

Animals

ICR male mice (8 weeks upon arrival; Harlan Laboratories, Indianapolis, IN) served as subjects. Mice were housed four per cage with ad libitum access to food and water on a 12-h light cycle in a humidity and temperature controlled vivarium that was approved by the Association for Assessment and Accreditation of Laboratory Animal Care. Mice received corn cob bedding and were fed Envigo Teklad mouse/rat diet 7102 (LM-485). Experiments were performed during the light cycle and were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University and followed the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Drugs

(−)-Nicotine hydrogen tartrate [(−)-1-methyl-2-(3- pyridyl) pyrrolidine (+)-bitartrate] and mecamylamine HCl (non-selective nAChR antagonist) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). PNU282987 (α7 nAChR agonist) and cocaine HCl were provided by the Drug Supply Program of the National Institute on Drug Abuse (Rockville, MD). Drugs were

dissolved in physiological saline and administered systemically (s.c. for nicotine, mecamylamine, PNU282987 and i.p. for cocaine). Fenofibrate (PPARα agonist), WY-14643 (PPARα agonist), and GW6471 (PPARα antagonist) and MK886 (PPARα antagonist) were purchased from Tocris (Minneapolis, MN) and dissolved in a mixture of 1:1:18 [1 volume ethanol/1 volume Emulphor-620 (Sanofi-Aventis, Bridgewater, NJ) and 18 volumes saline] and administered i.p. Drug solutions were prepared in 10 ml solutions (i.e. 3mg of drug in 10ml of vehicle indicates 3mg/kg dose). Freshly prepared solutions were injected at a total volume of 1 ml/100g of body weight. Doses are expressed as the free base of the drug.

Nicotine and cocaine conditioned preference studies

An unbiased CPP paradigm was performed as we previously described[80,284]. Briefly, the CPP apparatus consisted of three chambers in a linear arrangement (ENV3013; Med Associates, St Albans, VT). The external white and black chambers (20×20×20 cm each) differed in overall color and floor texture (white mesh or black rod), and were separated by a smaller gray chamber with a smooth PVC floor. Partitions could be removed to allow access from the gray chamber to the black and white chambers. On day 1 animals were confined to the middle chamber for a 5 min habituation and then allowed to freely move between all three chambers for 15 min. Time spent in each chamber was recorded and these data were used to populate groups of approximately equal bias in baseline chamber preference. Twenty-minute conditioning sessions occurred twice a day (days 2–4).

The nicotine group received nicotine in one large chamber and saline in the other large chamber. Treatments were counterbalanced to ensure some mice received the unconditioned stimulus in the morning and others received it in the afternoon. The nicotine paired chamber was randomized across groups. Sessions were 4 hr apart and were conducted by the same investigator. On test day (day 5) mice could access all chambers for 15 min in a drug free state. The preference score was calculated by determining the difference between time spent in the drug paired side on the test day versus the time in drug paired side on the baseline day. Any mouse showing preference for one side higher than 65% was not used in the study.

Nicotine Precipitated Withdrawal Studies

A well-established and validated nicotine withdrawal model was performed [89,94,182,297].Mice were infused with 24mg/kg/day nicotine or saline for 14 days using s.c. osmotic MPs (model 2000; Alzet Corporation, Cupertino, CA) implanted under isoflurane anesthesia [93].Nicotine concentration was adjusted according to animal weight and mini pump flow rate. On the morning of day 15 mice were pretreated with vehicle, WY-14643 (0.3, 1 and 5 mg/kg, i.p.; 15 min prior) or fenofibrate (50 and 100 mg/kg, i.p.;1 hr prior) before challenge with the nonselective nAChR antagonist mecamylamine (2 mg/kg; s.c.) to precipitate withdrawal. Affective (anxiety-like behavior) and physical (somatic signs and hyperalgesia) nicotine withdrawal signs were evaluated 10 min later as described in [93]. Mice were first evaluated for 5 min in the elevated plus maze test for anxiety-related behavior. Time spent on the open arms of the plus maze was used as a measure of anxiety-related response. The number of crosses between open and closed arms was counted as a measure of locomotor activity. The plus maze assessment was immediately followed by a 20 min observation of somatic signs measured as paw and body tremors, head

shakes, backing, jumps, curls and ptosis. Mice were placed in clear activity cages without bedding for the observation period. The total number of somatic signs was tallied for each mouse and the average number of somatic signs during the observation period was plotted for each test group. Hyperalgesia was evaluated using the hot plate test immediately following the somatic sign observation period. Mice were placed into a 10-cm wide glass cylinder on a hot plate(Thermojust Apparatus, Richmond, VA) maintained at 52°C. The latency to reaction time (jumping or paw licking) was recorded. The specific testing sequence was chosen based on our prior studies showing that this order of testing reduced within-group variability and produced the most consistent results [93]. All studies were performed by an observer blinded to experimental treatment.

Statistical analysis

Data were analyzed using the GraphPad software version 6.0 (GraphPad Software, Inc., La Jolla, CA) and expressed as the mean ± S.E.M. A one-way analysis of variance (ANOVA) in conjunction with Holm-Šídák comparison tests were conducted to determine significant effects of drug treatments vs controls. Two-way ANOVA followed by the Tukey multiple comparisons test was used in order to evaluate attenuation of dose response of nicotine CPP by PPARα agonist WY-14643. Comparisons were considered statistically significant when $p < 0.05$.

C. Results

Development of Nicotine CPP Attenuated by α7 nAChR Full Agonist PNU282987

Mice were conditioned with either saline or nicotine (0.5 mg/kg; s.c.) for 3 days in the CPP paradigm. The 0.5mg/kg dose of nicotine has been previously shown to produce a significant preference in the CPP test [37,78]. In Fig. 11 a robust CPP was observed in nicotine–conditioned mice pre-treated with vehicle [$F(4, 33) = 16.29$, $p < 0.0001$]. PNU282987 given 15 min prior to nicotine, reduced nicotine reward. As revealed by the Holm-Šídák comparison tests, PNU282987 (3mg/kg) significantly altered nicotine CPP ($p<0.05$), but was ineffective at the lower dose of 0.6mg/kg ($p>0.05$). PNU282987 at the dose of 3 mg/kg did not produce a preference in saline treated-mice.

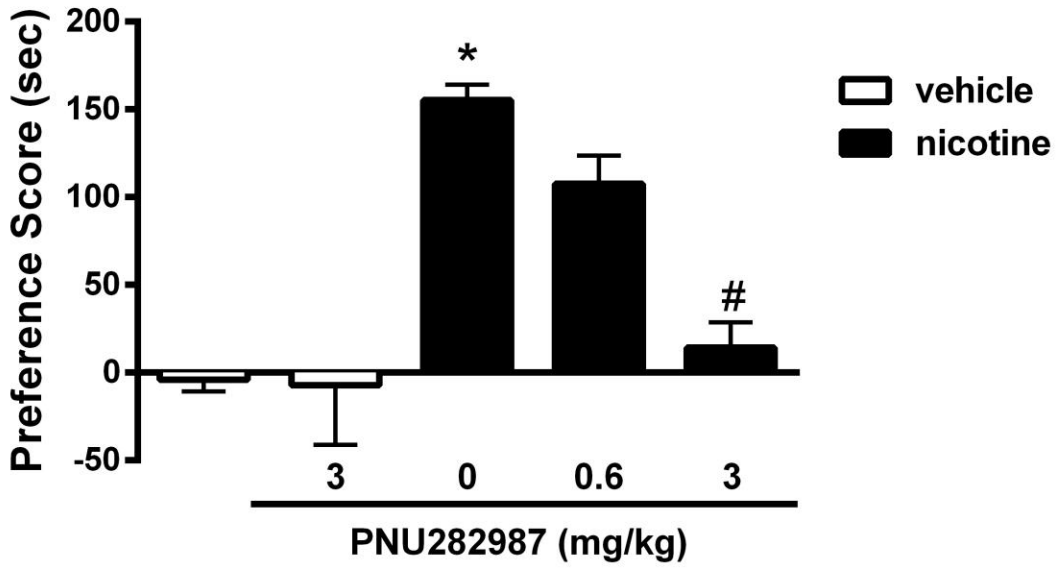


Figure 11: Attenuation of the Development of Nicotine CPP by α7 nAChR Orthosteric Full Agonist PNU282987

Mice were conditioned with either s.c. saline or nicotine (0.5mg/kg) for 3 days. A robust CPP was observed in nicotine-conditioned mice pre-treated with vehicle. The α7 agonist, PNU282987 (0.6 and 3 mg/kg; s.c.) reduced nicotine reward as measured by the CPP test. * Denotes $p<0.05$ from vehicle-vehicle. # Denotes $p<0.05$ from nicotine control. Each point represents the mean ± SEM of n=6-8 mice per group.

PPARα Antagonist Blocks α7 nAChR Agonist PNU282987 in Nicotine CPP

The PPARα antagonist GW6471 was utilized to evaluate the PPARα dependency of α7 nAChR activation in nicotine CPP. In Fig. 12 male ICR mice conditioned with 0.5mg/kg s.c. of nicotine for three days exhibited a significant preference [$F(7, 52) = 7.459$, $p<0.0001$]. One-way ANOVA revealed that pretreatment with the α7 nAChR agonist PNU282987 (3mg/kg; s.c.) given 15 min prior to nicotine attenuated nicotine CPP. This attenuation was significantly blocked by the PPARα antagonist GW6471 (2mg/kg; i.p) administered 30 min prior to PNU282987 ($p<0.05$), whereas GW6471 did not have an effect on nicotine CPP ($p>0.05$). PNU282987 and GW6471 did not cause aversion or preference on their own or in combination.

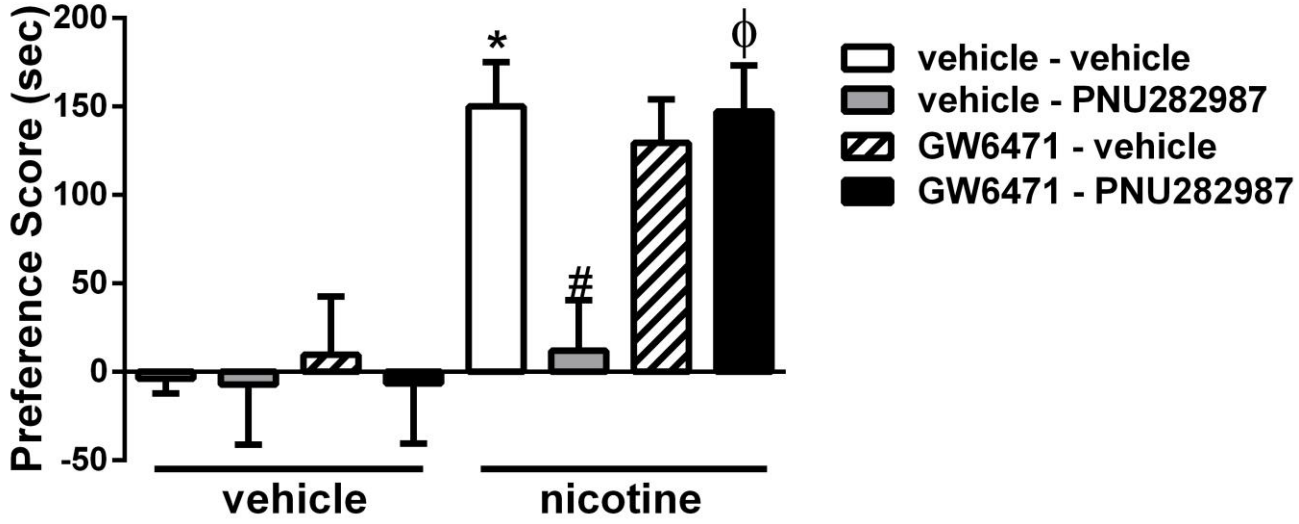


Figure 12: Interaction between PPARα and α7 nAChR in the Nicotine Reward.

Mice were conditioned with either s.c. saline or nicotine (0.5mg/kg) for 3 days. A robust CPP was observed in nicotine-conditioned mice pre-treated with vehicle. The α7 agonist PNU282987 (mg/kg; s.c.) reduced nicotine reward. The PPARα antagonist GW6471 (2 mg/kg; i.p.) blocked the effect of the α7 nAChR agonist in nicotine CPP. * Denotes $p<0.05$ from vehicle-vehicle; # denotes $p<0.05$ from nicotine control. Φ Denotes $p<0.05$ from vehicle-PNU282987-nicotine. Each point represents the mean ± SEM of n=6-9 mice per group

The PPARα Agonist WY-14643 Attenuated Nicotine CPP

We then tested the impact of direct activation of PPARα using the selective and potent PPARα agonist WY-14643 on nicotine CPP. Mice were conditioned with either saline or nicotine (0.5 mg/kg) for 3 days in the CPP paradigm. In Fig. 13 a robust CPP was observed in nicotine conditioned mice pre-treated with vehicle [$F (5, 36) = 26.27$, $p<0.0001$]. WY-14643 reduced nicotine reward in a dose-dependent manner at all doses tested (0.3, 0.6 and 1 mg/kg) ($p<0.05$). On its own WY-14643 did not produce a preference or aversion in saline treated mice.

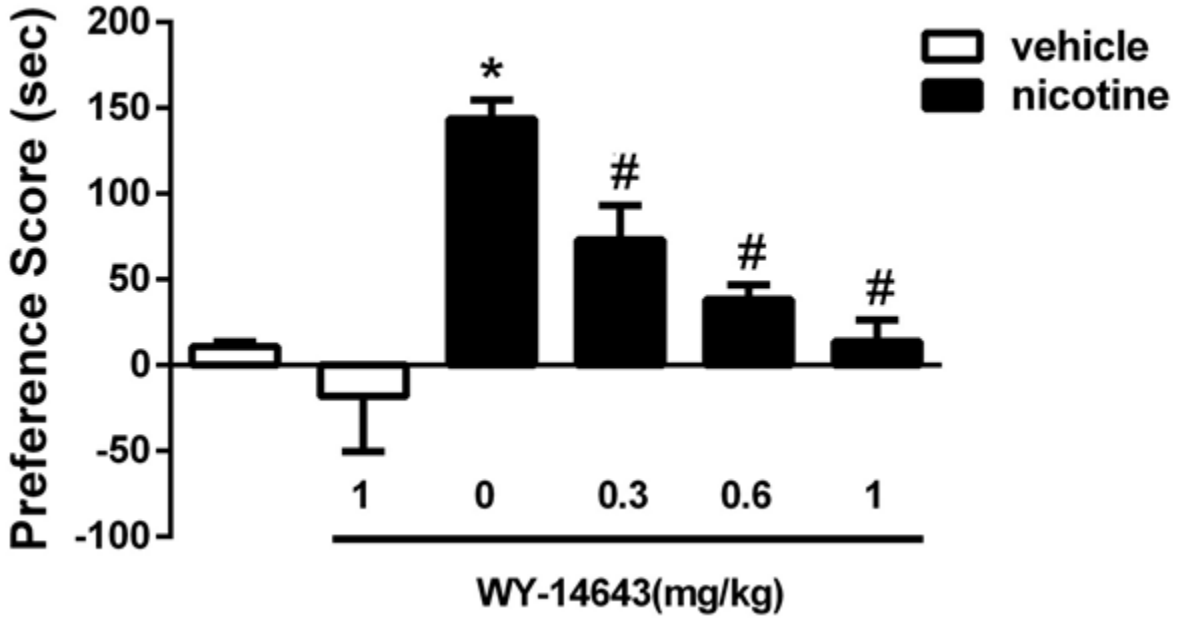


Figure 13. PPARα Agonist WY-14643 Attenuated Nicotine CPP.

Mice were conditioned with either s.c. saline or nicotine (0.5 mg/kg) for 3 days. A robust CPP was observed in nicotine-conditioned mice pre-treated with vehicle. An i.p. injection of PPARα agonist WY-14643 (0.3, 0.6, and 1 mg/kg) reduced nicotine reward as measured by the CPP test. *Denotes $p<0.05$ from vehicle control; #Denotes $p<0.05$ from nicotine control. Each point represents the mean ± SEM of n=6-8 mice per group.

The PPARα Antagonist Blocked the Effects of the PPARα Agonist WY-14643 in Nicotine CPP

The PPARα antagonist, MK886, was used to investigate the PPARα-dependency of WY-14643. At the highest effective dose, the PPARα agonist WY-14643 (1mg/kg; i.p.) significantly reduced nicotine preference [$F(2, 19) = 46.40$, $p < 0.0001$] (Fig. 14) and the PPARα antagonist MK886 (6mg/kg; i.p.), given 30 min prior to WY-14643 in the nicotine CPP test, completely blocked the effect of WY-14643 ($p<0.05$). WY-14643 and MK886 did not produce a preference or aversion in saline treated-mice [$F(2, 17) = 0.9040$, $p < 0.4235$].

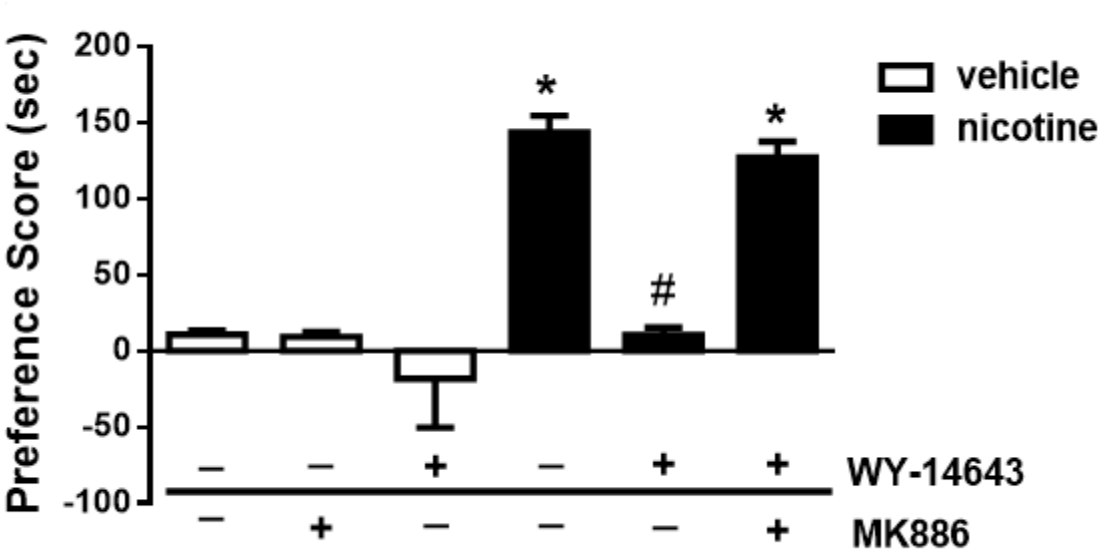


Figure 14. The Effect of PPARα Antagonist MK886 on WY-14643 in Nicotine CPP.

Mice were conditioned with either s.c. saline or nicotine (0.5 mg/kg) for 3 days. A robust CPP was observed in nicotine-conditioned mice pre-treated with vehicle The PPARα antagonist MK886 (6 mg/kg; i.p.) blocked the effect of WY-14643 (1 mg/kg, i.p.) in nicotine CPP.* Denotes $p<0.05$ from vehicle control; # Denotes $p<0.05$ from nicotine control. Each point represents the mean ± SEM of n=6-8 mice per group.

WY-14643 Did Not Shift the Potency of Nicotine in Nicotine CPP

To test the effect of the PPARα agonist WY-14643 on the potency of nicotine in the CPP test WY-14643 (1 mg/kg; i.p.) was administered 15 minutes prior to nicotine (0.1, 0.5 and 1 mg/kg; s.c.) in the CPP test. Two-way ANOVA revealed that a significant nicotine preference [$F(3, 53) = 9.225$, $p < 0.0001$], a significant blockage of nicotine preference by WY-14643 [$F(1, 53) = 44.54$, $p < 0.0001$] and interaction [$F(3, 53) = 4.315$, $p = 0.0085$]. In Fig. 15 nicotine preference was significant at 0.5 and 1mg/kg doses after 3 days of conditioning ($p < 0.001$). WY-14643 pretreatment significantly attenuated nicotine preference at 0.5 and 1mg/kg ($p < 0.05$) and had no effect on the 0.1 mg/kg dose of nicotine ($p > 0.05$). WY-14643 did not produce preference or aversion on its own ($p > 0.05$).

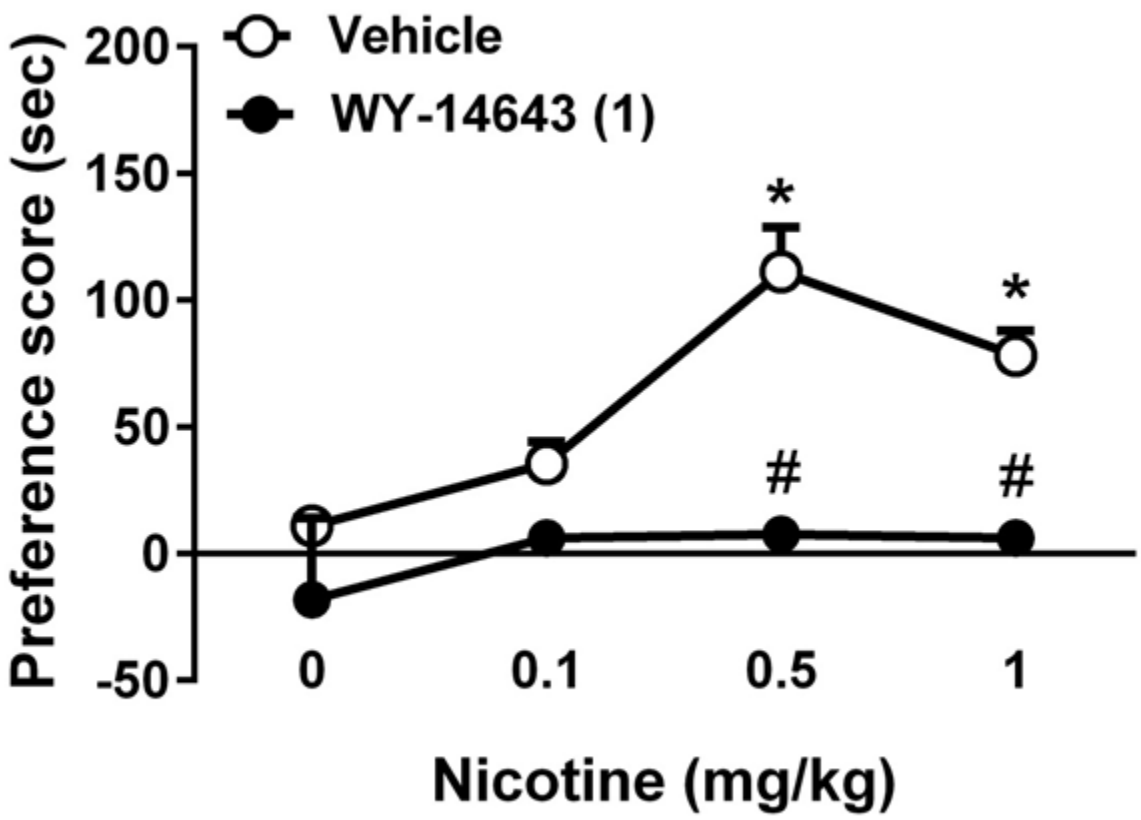


Figure 15. WY-14643 Attenuated Multiple Doses of Nicotine in the CPP test.

To evaluate blockade of dose response of nicotine CPP by PPARα agonist mice were conditioned with either saline or nicotine (0.1, 0.5 and 1 mg/kg; s.c.) for 3 days. A robust CPP was observed in nicotine-conditioned mice pre-treated with vehicle by the dose of 0.5 mg/kg or above. Pretreatment with WY-14643 (1 mg/kg; i.p.) reduced nicotine-CPP at the dose of 0.5 and 1 mg/kg nicotine. * Denotes $p<0.05$ from vehicle control; # Denotes $p<0.05$ from nicotine control. Each point represents the mean ± SEM of n=6-8 mice per group.

PPARα Agonist WY-14643 Did Not Attenuate Cocaine CPP

To test for the behavioral selectivity of WY-14643 on nicotine CPP, WY-14643 was evaluated in cocaine CPP as previously described[139,298]. In Fig. 16 robust preferences for cocaine (10mg/kg; i.p.) and nicotine (0.5mg/kg; s.c.) were produced after 3 days of conditioning in mice [$F(4, 32) = 32.63$, $p < 0.0001$]. The 10 mg/kg dose of cocaine has been previously shown to produce a significant preference in the CPP test [159,299]. Although WY-14643, with a 15 min pretreatment, totally reduced nicotine reward at 1mg/kg as previously observed in this study ($p<0.05$) , it had no significant effect on cocaine preference ($p>0.05$).

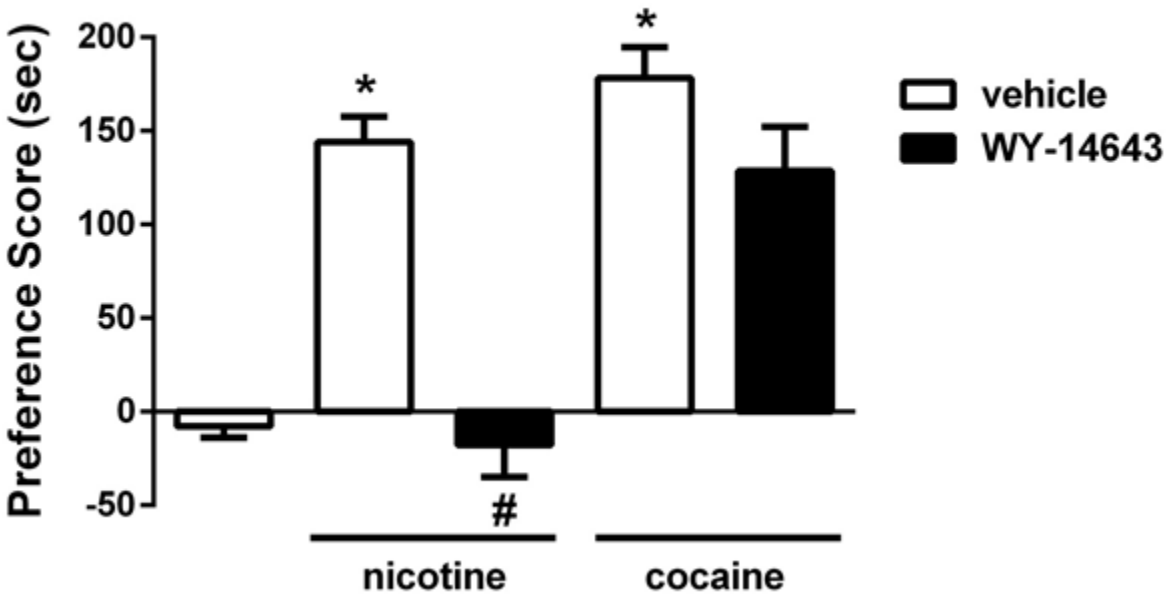


Figure 16. Effects of PPARα Agonist WY-14643 on Nicotine and Cocaine CPP.

To test the selectivity of the attenuating effect of the PPARα agonist in nicotine CPP a separate group of mice was conditioned with saline, cocaine (10 mg/kg; i.p.) or nicotine (0.5 mg/kg; s.c.) for 3 days. A robust CPP was observed in both nicotine conditioned and cocaine-conditioned mice pre-treated with vehicle. The PPARα agonist WY- 14643 (1mg/kg; i.p.) reduced nicotine reward, but not cocaine reward as measured by the CPP test. * Denotes $p<0.05$ from vehicle control; # Denotes $p<0.05$ from nicotine control. Each point represents the mean ± SEM of n=6-8 mice per group

Clinically Used PPARα Agonist Fenofibrate Reduced Nicotine CPP

We utilized the clinically available PPARα agonist fenofibrate in the nicotine CPP paradigm. As previously observed in this study one way ANOVA showed that nicotine induced a significant preference in comparison to saline-treated mice after the 3-day conditioning period [$F(6, 45) = 4.078$, $p=0.0024$]. In Fig. 17 pretreatment with lower doses of fenofibrate (1 and 9 mg/kg) 1hr prior to nicotine did not significantly alter nicotine CPP ($p>0.05$). However, the dose of 50 mg/kg of fenofibrate reduced nicotine preference significantly ($p<0.05$). The effect of fenofibrate was loss at 100mg/kg ($p>0.05$). Fenofibrate had no effect on its own in saline treated-mice. Fenofibrate was administered at doses previously described [291,292].

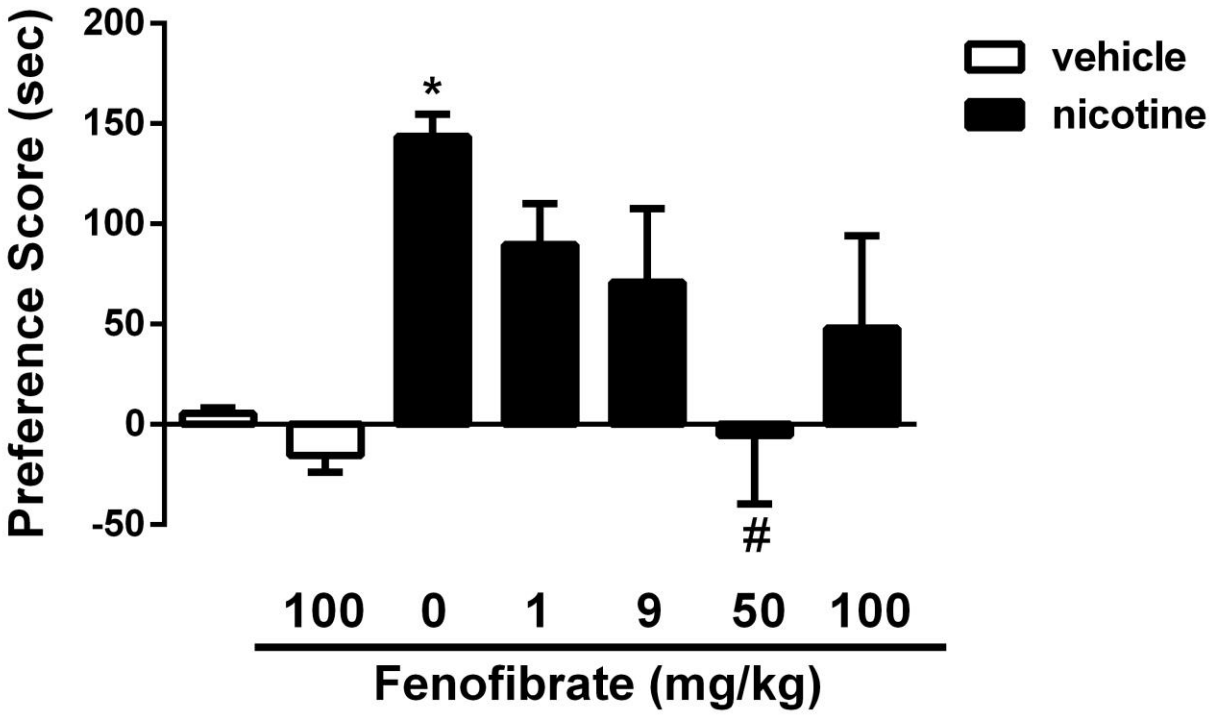


Figure 17. Effect of PPARα Agonist Fenofibrate on Nicotine CPP

Mice were conditioned with either s.c. saline or nicotine (0.5mg/kg) for 3 days. A robust CPP was observed in nicotine conditioned mice pre-treated with vehicle. Fenofibrate (1, 9,50 and 100 mg/kg; i.p.), clinically used PPARα agonist, reduced nicotine reward as measured by the CPP test. *Denotes $p<0.05$ from vehicle control; # Denotes $p<0.05$ from nicotine control. Each point represents the mean ± SEM of n=6-8 mice per group.

The PPARα Antagonist Did Not Block Fenofibrate in Nicotine CPP

The PPARα antagonist, MK886, was also used to investigate the PPARα-dependency of fenofibrate. MK886 was used to be consistent with previous studies utilizing another PPARα agonist of the fibrate family, clofibrate in nicotine reward [34]. At the highest effective dose used fenofibrate (50mg/kg; i.p.), with 1 hr pretreatment, significantly attenuated nicotine preference [$F(5, 36) = 3.835$, $p = 0.0069$] (Fig. 18), but the PPARα antagonist MK886 (6mg/kg; i.p.) had no effect on fenofibrate in nicotine CPP ($p>0.05$, Fig.18). Fenofibrate and MK886 did not produce a preference or aversion in saline treated-mice.

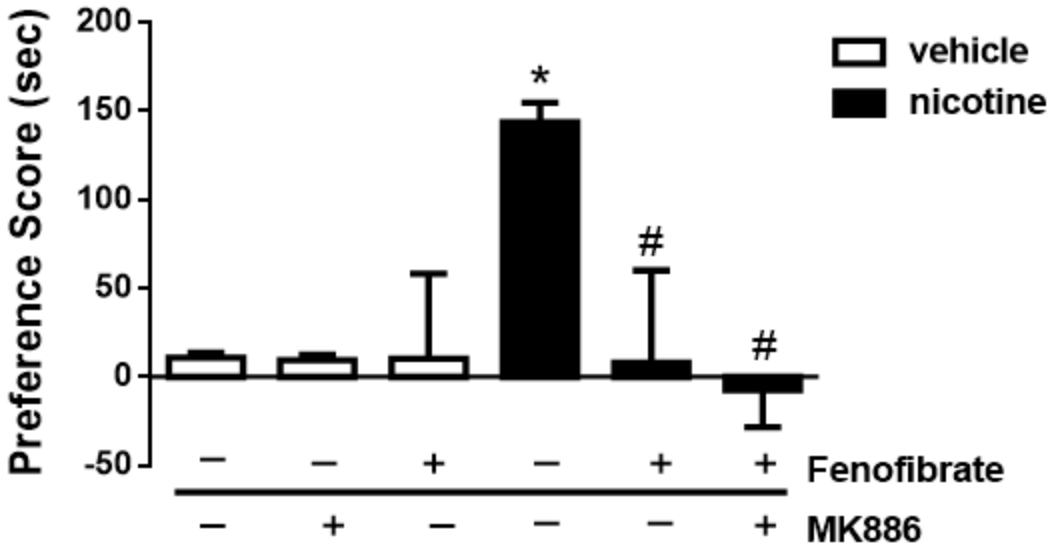


Figure 18. The Effect of PPARα Antagonist MK886 on Fenofibrate in Nicotine CPP

Mice were conditioned with either s.c. saline or nicotine (0.5mg/kg) for 3 days. A robust CPP was observed in nicotine-conditioned mice pre-treated with vehicle. The PPARα antagonist MK886 (6 mg/kg; i.p.) did not block the effect of fenofibrate (50mg/kg; i.p.) in nicotine CPP. * Denotes $p<0.05$ from vehicle control; # Denotes $p<0.05$ from nicotine control. Each point represents the mean ± SEM of n=6-8 mice per group.

Nicotine Withdrawal Signs Attenuated by PPARα Agonist WY-14643

The physical (somatic signs and hyperalgesia) and affective (anxiety-related behavior) signs of nicotine withdrawal were measured in mice following pretreatment with either WY-14643 or vehicle 15 min prior to mecamylamine administration on day 15. In Fig 19 nicotine withdrawn mice had a significantly increased anxiety-related behavior in the plus maze[$F (5, 32) = 4.853$, $p=0.0020$] (Fig. 19A), increased expression of somatic withdrawal signs[$F (5, 33) = 24.04$, $p<0.0001$] (Fig. 19B) and decreased response latencies in the hot-plate test [$F (5, 34) = 3.432$, $p=0.0129$] (Fig.19C) compared to control mice implanted with saline MPs. In Fig. 19A one-way ANOVA revealed that pretreatment with WY-14643 attenuated anxiety-like behavior (time in open arms in the plus-maze test) at the dose of 5 mg/kg ($p<0.05$). In addition, as shown in Fig. 19B pretreatment with 1 and 5 mg/kg of WY-14643 decreased nicotinic somatic withdrawal signs ($p<0.05$). In our study somatic signs were expressed as followed: paw tremors (~70%), body tremors (~5%), head shakes (~10%), backing (~15%). WY-14643 reduced these individual somatic signs in a uniformed manner. Finally, in Fig. 19C pretreatment with WY-14643 also attenuated the expression of hyperalgesia (hot-plate latency) at 5 mg/kg ($p<0.05$). The highest dose of WY-14643 tested (5 mg/kg) did not significantly affect behavioral responses in saline-infused mice in any withdrawal test.

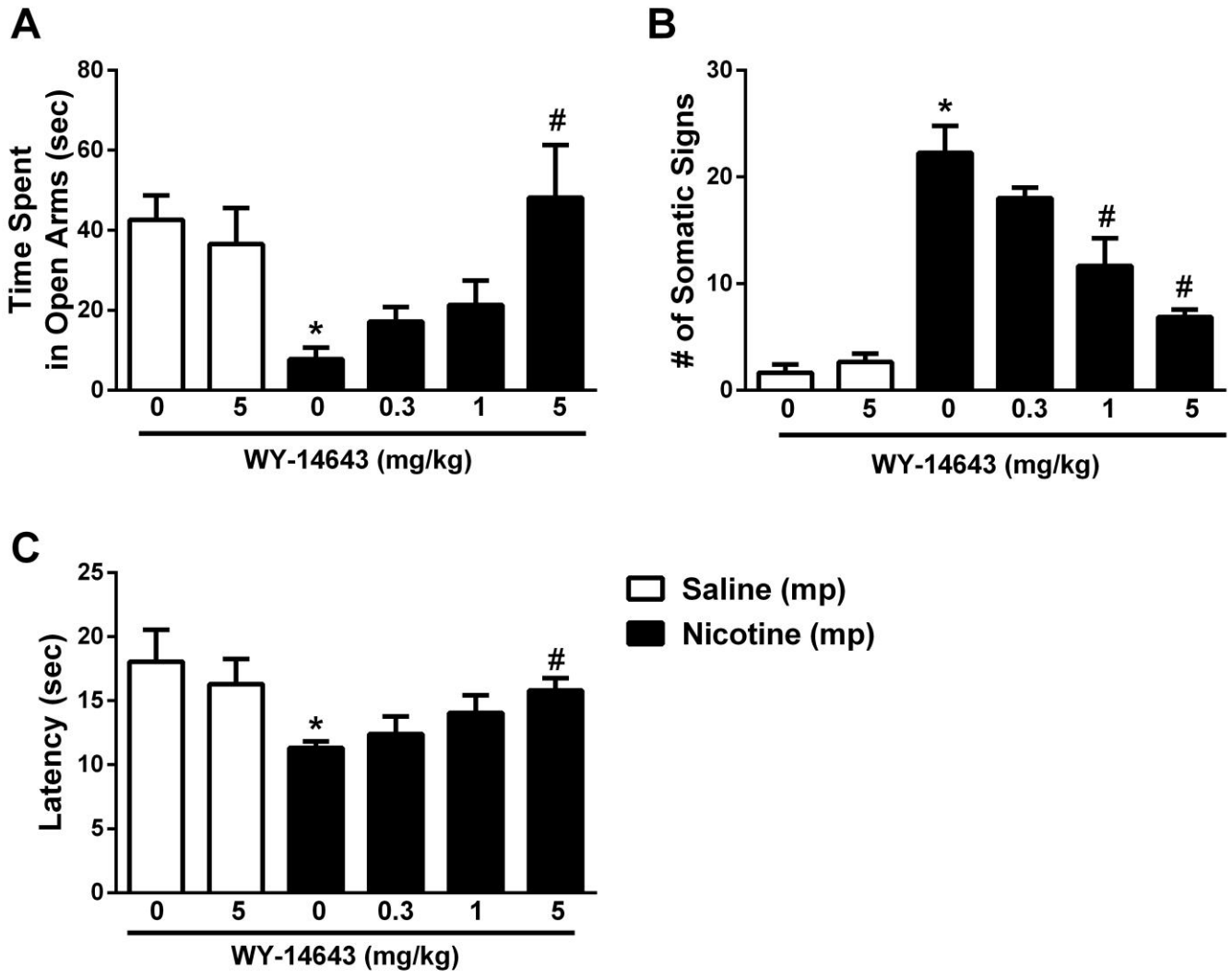


Figure 19: Effects of PPARα Agonist WY-14643 on Physical and Affective Signs of Precipitated Nicotine Withdrawal.

Mice were chronically infused with saline or nicotine (24 mg/kg/day) for 14 days. On day 15 mice received i.p. injection of WY-14643 (0.3, 1 and 5 mg/kg) or vehicle. Mice then were administered mecamylamine (2mg/kg; s.c.) 10 min prior to behavioral assessment of **A**) anxiety-like behaviors (Time spent in the open arm**), B)** somatic signs, and **C)** hyperalgesia (hot plate latency).Nicotine induced withdrawal symptoms: increased anxiety related behavior and somatic signs, but decreased hot plate latency. Compared to vehicle, pretreatment with WY-14643**: A)** attenuated the anxiety-like behavior at 5mg/kg; **B)** reduced somatic signs at 1 and 5mg/kg; and **C)** significantly increased hot plate latency at 5mg/kg in nicotine withdrawn mice. Each point represents the mean ± S.E.M. of n=6–8 mice per group. * Denotes $p < 0.05$ vs. Saline MP group, # Denotes $p < 0.05$ vs. Nicotine MP group.

PPARα Agonist WY-14643 Did Not Alter Arm Crosses in the Elevated Plus Maze.

To examine whether or not the results observed in the elevated plus maze test was possibly confounded by alterations in locomotor activity induced by WY-14643 administration, the number of arm crosses were recorded. As shown in Table 5 WY-14643 had no effect on the number of arm crosses in the plus maze [$F(5, 32) = 0.4386$, $p=0.8182$].

Table 5: WY-14643 does not significantly alter the average number of arm crosses in the elevated plus maze test

Mice undergoing nicotine withdrawal received WY-14643(0.3,1, 5; i.p.) or vehicle. The average number of arm crosses were recorded in the plus maze test. The numbers are presented as the total number of arm crosses ± SEM (n=6-8).

Treatment	**Average number of arm crosses ±SEM**
Saline MP-vehicle	**7.8± 0.9**
Saline MP- WY-14643 (5)	**8±0.8**
Nicotine MP-vehicle	**7.1±0.4**
Nicotine MP-WY-14643 (0.3)	**7.2±0.3**
Nicotine MP-WY-14643 (1)	**7.2±0.3**
Nicotine MP-WY-14643 (5)	**7.7±0.5**

Fenofibrate Modestly Attenuated Nicotine Withdrawal

Fenofibrate was administered 1 hr prior to mecamylamine on day 15 after 14 days of continuous nicotine exposure via osmotic MPs. Following mecamylamine administration nicotine withdrawals signs (anxiety-like behavior, somatic signs and hyperalgesia) were measured in mice. In Fig. 20 nicotine withdrawn mice displayed an increase in anxiety-related behavior in the plus maze [$F (5, 42) = 22.08$, $p<0.0001$] (Fig. 20A), enhanced expression of somatic withdrawal signs [$F (5, 42) = 63.26$, $p<0.0001$] (Fig. 20B) and attenuated response latencies in the hot-plate test [$F (5, 42) = 12.12$, $p<0.0001$](Fig. 20C) in comparison to their saline MP-implanted counterparts. In Fig. 20A one way ANOVA revealed that pretreatment with fenofibrate had no effect on anxiety-like behavior (time in open arms in the plus-maze test) at both doses tested (50 and 100 mg/kg) ($p>0.05$). However, as shown in Fig. 20B pretreatment with fenofibrate partially attenuated nicotinic somatic withdrawal signs only at the highest dose used of 100 mg/kg ($p<0.05$). Somatic signs were expressed in the following distribution: paw tremors (~70%), body tremors (~5%), head shakes (~10%), backing (~15%). Fenofibrate partially attenuated these individual somatic signs in a uniformed manner. Lastly, as shown in Fig. 20C pretreatment with fenofibrate was ineffective at attenuating the expression of hyperalgesia (hot-plate latency) at both doses tested ($p>0.05$). The highest dose of fenofibrate tested (100 mg/kg) did not significantly affect behavioral responses in saline infused mice in any withdrawal test. In the nicotine withdrawal studies fenofibrate was administered at doses previously described [291,292].

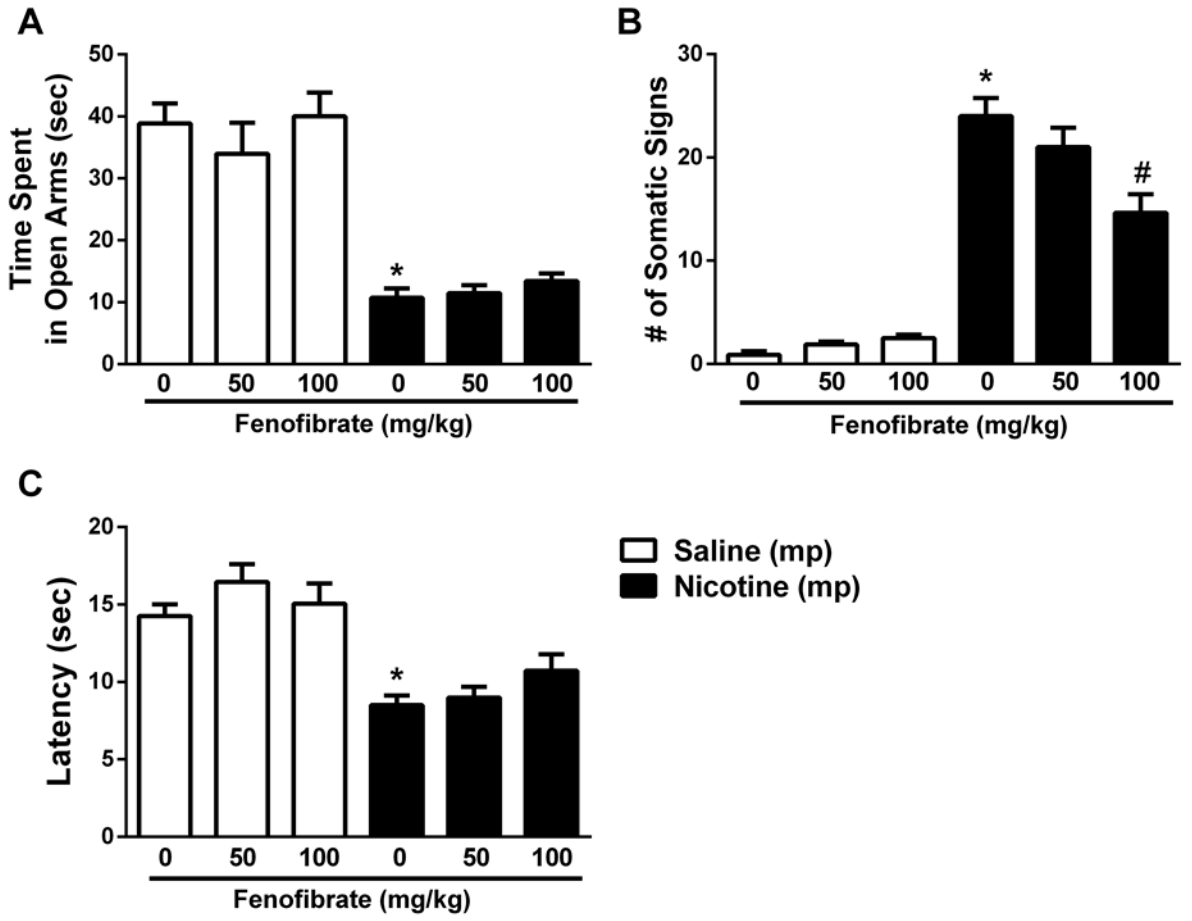


Figure 20: Effects of PPARα Agonist Fenofibrate on Physical and Affective Signs of Precipitated Nicotine Withdrawal.

Mice were chronically infused with saline or nicotine (24 mg/kg/day) for 14 days. On day 15mice received fenofibrate 1 hr pretreatment (50 and 100 mg/kg; i.p.) or vehicle. Withdrawal was precipitated by administration of mecamylamine (2mg/kg; s.c.) 10 min prior to behavioral testing of: **A)** anxiety-like behaviors (Time spent in the open arm**); B)** somatic signs; and **C)** hyperalgesia (hot plate latency). Nicotine induced withdrawal symptoms increase anxiety-related behavior and somatic signs, but decrease hot plate latency. Compared to vehicle, pretreatment with fenofibrate: **A)** had no effect on the anxiety-like behavior; **B)** reduced somatic signs at 100 mg/kg; and **C)** did not alter hot plate latency in nicotine withdrawn mice. Each point represents the mean ± S.E.M. of 8 mice per group. * Denotes $p < 0.05$ vs. Saline MP group, # Denotes $p < 0.05$ vs. Nicotine MP group

PPARα Agonist Fenofibrate Did Not Alter Arm Crosses in the Elevated Plus Maze.

To examine whether or not the results observed in the elevated plus maze test was possibly confounded by alterations in locomotor activity induced by fenofibrate administration, the number of arm crosses were recorded. As shown in Table 6 fenofibrate did not significantly alter the number of arm crosses in the plus maze test [$F(5, 42) = 0.5318$, $p = 0.7509$].

Table 6: Fenofibrate does not have an effect on the average number of arm crosses in the elevated plus maze test

Mice undergoing nicotine withdrawal received fenofibrate (50 and 100 mg/kg; i.p.) or vehicle. The average number of arm crosses were recorded in the plus maze test. The numbers are presented as the total number of arm crosses ± SEM (*n*=8).

Treatment	**Average number of arm crosses ±SEM**
Saline MP-vehicle	**8.3± 0.6**
Saline MP- Fenofibrate (50)	**7.6±0.5**
Saline MP- Fenofibrate (100)	**7.4±0.3**
Nicotine MP-vehicle	**7.1±0.5**
Nicotine MP-Fenofibrate (50)	**7.8±0.5**
Nicotine MP-Fenofibrate (100)	**8±0.8**

D. Discussion

This is the first report demonstrating the ability of a PPARα antagonist to block the inhibitory effects of an α7 nAChR agonist on nicotine reward in a mouse CPP paradigm (Fig. 12). This suggests that α7 nAChR activation attenuates nicotine CPP in a PPARα-dependent mechanism. We therefore compared the effects of a selective and potent PPARα agonist, WY-14643, to fenofibrate, a clinically available PPARα agonist in nicotine mouse models of reward and withdrawal. Our results provide some important and novel insights about the effects of PPARα agonists in these nicotine dependence tests. The PPARα agonists WY-14643 and fenofibrate attenuated nicotine preference as expected but fenofibrate was less potent (Fig. 13 and Fig.17). In addition, the attenuation by fenofibrate in nicotine CPP was not PPARα-mediated (Fig.18). Also, in contrast to WY-14643, fenofibrate had a modest efficacy in reducing nicotine withdrawal signs (Fig. 19 and Fig. 20).

Our results indicated that attenuation by α7 nAChR activation in nicotine CPP is PPARα mediated (Fig. 12). This finding is consistent with the suggestion that an α7 nAChR agonist prevents nicotine-induced excitation of dopamine neurons via PPARα mechanism [116]. Indeed, the PPARα agonist WY-14643 completely and dose-dependently blocked nicotine conditioned reward in the CPP test (Fig. 13). In addition, WY-14643 at the highest effective dose (1 mg/kg) blocked all doses of nicotine in the CPP test (Fig. 15). Furthermore, WY-14643 (1mg/kg) had no significant effect on cocaine CPP suggesting behavioral selectivity of WY-14643 for attenuating nicotine reward (Fig.16). In support of our findings WY-14643 has been previously shown to be ineffective in reducing cocaine self-administration [277]. Our findings with WY-14643 are consistent with other PPARα agonists such as clofibrate that was reported to attenuate nicotine reinforcement and reinstatement in rats through a PPARα mechanism of action [34,154,277]. Our

study with fenofibrate in nicotine CPP produced novel findings. Fenofibrate blocked the development of nicotine CPP at a lower potency (a 9-fold difference estimate) than WY-14643, the selective and potent PPARα agonist (Fig. 17). In fact, the dose of fenofibrate to completely block nicotine CPP was 50mg/kg. At the higher dose of 100 mg/kg, fenofibrate-treated mice were no longer statistically different from the nicotine-treated mice. Contrary to WY-14643, fenofibrate blockade of nicotine preference was not PPARα-mediated. The PPARα antagonist MK886, blocked the effects of WY-14643 but not fenofibrate in the nicotine CPP test (Fig. 14 and Fig.18). This is in contrast with the effects of another member of the fibrate family, clofibrate, as well as other PPARα agonists such WY-14643 and methOEA in i.v. nicotine self-administration and reinstatement models in rats and primates [34,277]. Indeed, the reduction of nicotine reinforcement by these PPARα agonists was blocked by MK886. The lack of a PPARα-dependency in the effect of fenofibrate is not entirely surprising since it has also been reported in anti-proliferative and anti-inflammatory *in vitro* studies [300–303].

Our nicotine withdrawal results suggest PPARα activation by WY-14643 is effective at attenuating nicotine withdrawal signs in a mouse model. To our knowledge this is the first study to evaluate PPARα agonists in a preclinical test for nicotine withdrawal. WY-14643 attenuated both the affective (anxiety-like behavior) and physical (somatic and hyperalgesia) signs of withdrawal (Fig. 19) whereas fenofibrate only partially and modestly reduced the somatic signs intensity at the highest dose used, 100 mg/kg (Fig. 20). Higher doses of fenofibrate were not investigated due to adverse locomotor effects (data not shown). Clinically available smoking cessation therapies act to a large extent by reducing the nicotine withdrawal signs/symptoms [82], one of the primary causes of high tobacco relapse rates [29]; consequently, our animal studies included a focus on nicotine withdrawal. Somatic signs have shown to contribute less to

nicotine-seeking behavior than affective signs [108,169]; thus, the modest reduction of somatic signs by fenofibrate may not predict its efficacy as a smoking cessation aid.

The α7 nAChR full agonist PNU282987 used in the CPP studies is selective for the α7 nAChR [248,304,305]. However, it has been suggested that α7 nAChR activation might indirectly lead to downregulation of β2-nicotinic subunits via PPARα-induced phosphorylation of these subunits [116,278]. Indeed, α7 nAChR pharmacological activation by PNU282987 enhanced the neuronal levels of endogenous PPARα ligands OEA and PEA in the VTA [116]. Therefore, PPARα activation by WY-14643 may attenuate nicotine conditioned reward in the CPP test via a similar mechanism leading to a functional downregulation of β2 subunits. β2-containing nAChRs are well known to play an important role in nicotine reward in the CPP test [37].The lack of reduction of cocaine CPP by PPARα agonist WY-14643 is somewhat surprising if we assume an important role for β2-containing nAChRs in the effect of PPARα activation. Nevertheless, it is possible that this mechanism (i.e. β2- containing nAChR downregulation) may not be involved in cocaine CPP. Unlike nicotine CPP, genetic and pharmacological activation of α7 nAChRs does not alter cocaine preference[35]. It has been reported that cocaine CPP is partially reduced in β2 knockout mice[298] at 5mg/kg of cocaine, suggesting that β2- containing nAChRs play a role in cocaine CPP. However, at the higher dose of 10mg/kg, the dose used in our study, no reduction of cocaine CPP was observed[298]. Another possibility is the degree of phosphorylation of the β2 subunit may not be sufficient enough to alter cocaine CPP in comparison to a complete genetic ablation of the β2 subunit (β2 knockout mice). Therefore, the proposed mechanism of α7 nAChR activation indirectly downregulating β2-containing nAChRs may not play a role in cocaine CPP. In nicotine withdrawal, it is possible that regulation of β2 nAChR subunits influences the reversal of nicotine withdrawal-related signs by the PPARα agonist WY-14643. Indeed, β2-containing

nAChRs are important for the affective signs of nicotine withdrawal [93]. In addition, animal studies reported a correlation between the time-course of brain β2-containing nAChRs upregulation and nicotine withdrawal signs[306]. Furthermore, nicotine withdrawn smokers have upregulated β2-containing nAChRs [195].

Collectively our preclinical findings on fenofibrate are consistent with its lack of effectiveness seen in a recent clinical study [307] as a smoking cessation aid. The pilot study was a 4-week evaluation of fenofibrate using a within-subjects crossover design with nicotine-dependent volunteers (n=38). Although that experiment had limitations in sample size, duration and used only one dose of fenofibrate, our data suggest that fenofibrate might not be the appropriate PPARα drug to use because it has modest effects on nicotine withdrawal and has been shown to be a weak and non-selective PPARα agonist (EC_{50} >10 μM) [294,295]. Importantly, our data with WY-14643 and those reported with clofibrate[34] suggests that PPARα is a potential molecular target to evaluate for smoking cessation. Notably, PPARαs undergo different structural conformations upon interaction with different ligands and each ligand-receptor conformation may lead to different patterns of gene expression modulation. For example activation of PPARα by WY-14643 and fenofibrate activate different set of genes as well a small set of overlapping genes [308]. Therefore, evaluation of more selective and potent PPARα agonists such as LY518674 (>2000-fold more potent and >300-fold more selective than fenofibrate) and PPARα biased agonists such as the selective PPAR modulators (SPPARMS) K-877 (Pemafibrate®) [309] should be considered. SPPARMS are thought to interact with the large binding pocket of PPARα to induce a different co-factor recruitment, resulting in higher potency and fewer adverse side effects than the original fibrate compounds [310]. LY518674 and K-877 are currently in phase II trials with promising results in treating dyslipidemia [311,312]. These compounds may prove to be

more efficacious candidates for smoking cessation therapy; however, preclinical studies are imperative to investigate this hypothesis. In summary, our findings build on the understanding of the underlying mechanism of α7 nAChR activation in nicotine reward. Further investigation needs to be conducted to elucidate the role of PPARα mediation of α7 nAChR in nicotine dependence.

CHAPTER FOUR

Investigating the Role of Ethanolamides in Nicotine Dependence

A. Introduction

Our results from the previous chapter suggested that fenofibrate, a PPARα agonist currently used to reduce high cholesterol levels, might not be an efficacious treatment for nicotine dependence. Fenofibrate reduced nicotine reward in the CPP test at the high dose of 50mg/kg in a non-PPARα mediated manner. In addition, fenofibrate showed a very modest efficacy on nicotine withdrawal. Our results with fenofibrate are in agreement with a clinical study that showed fenofibrate was ineffective as a smoking cessation aid [307]. We suggested that this lack of efficacy in rodents and human testing is probably due to the fact that fenofibrate is weak and non-selective activator of PPARα [295,313] [295,314]. In addition, PPARα expression in the brain is lower than in other organs such as the liver where it induces its lipolysis effects [287,315]. This suggests that attenuation of nicotine dependence may require the use of higher potency and efficacy PPARα agonists. Indeed, in the previous chapter, we showed that in contrast to fenofibrate, the potent and selective PPARα agonist WY-14643 attenuated nicotine CPP in a PPARα-dependent manner and reversed nicotine withdrawal signs in our models. Thus, PPARα may still be a viable target for smoking cessation but it is clear fenofibrate is not a desirable PPARα agonist to use.

The nuclear receptor PPARα is a transcription factor that mediates the transcription of genes involved in inflammation and lipolysis [276]. Of importance, PPARαs are located in brain regions associated with reward [287–289] and activated by endogenous ligands OEA and PEA. Recent

evidence showed that exposure to nicotine may regulate the endogenous PPARα system. For example, a reduction in the levels of the endogenous PPARα agonist OEA was observed in the VTA dialysate of rats under a nicotine i.v. self-administration regimen [316], suggesting that the ethanolamide deficit may contribute to nicotine dependence. Therefore, correcting this deficit by enhancing the levels of endogenous PPARα agonist may be a potential approach to treat nicotine dependence.

Direct administration of OEA and PEA to activate PPARα may be one possible strategy. It has been previously shown that methOEA (a long-lasting analog of OEA) reduces the rewarding effect of nicotine in intravenous self-administration after systemic administration in rats [277]. Although providing proof of principle, utilizing natural lipids as therapeutic agents has limitations. such as fast metabolism, poor pharmacokinetic properties upon oral ingestion in humans [317,318]. Inhibiting the degradative enzymes of OEA and PEA may serve as an alternative strategy to increase endogenous OEA and PEA activity at PPARα. Indirect activation of a receptor bypasses overstimulation of the system, which attenuates unintended side effects. In addition, inhibiting the degradative enzyme instead of direct OEA/PEA administration restricts the effect of the drug only to locations that possess that particular enzyme which also reduces unwanted side effects that may be caused by widespread activation of OEA/PEA targets after their administration. This is a similar approach that has been used in the cannabinoid field where indirect activation of cannabinoid receptors, by inhibiting degradative enzymes such as FAAH and MAGL, has been shown to produce more therapeutic benefits and bypass some negative side effects of direct agonists[201,319]. OEA and PEA are enzymatically degraded by FAAH and the lysosomal enzyme N-acylethanolamine hydrolyzing acid amidase (NAAA) [320,321] which are enzymes that differ in their catalytic mechanisms, structure, and selectivity for substrates. Both

enzymes have been shown to enhance OEA and PEA levels [320,322,323], but inhibition of FAAH also increases AEA levels, one of the endogenous cannabinoids. FAAH has more selectivity for AEA in comparison to PEA and OEA, whereas NAAA is more selective for PEA than AEA [321]. Therefore, the inhibition of FAAH is not a favorable approach to enhance OEA and PEA levels in hopes of reducing the rewarding effect of nicotine. NAAA is expressed in regions of the brain associated with reward along with PEA, OEA, and PPARα [287,289,321,324]. NAAA inhibition enhances OEA and PEA levels [325], and both OEA and PEA have been shown to block nicotine-induced VTA dopaminergic neuron excitation in a PPARα dependent manner [115]. Thus we hypothesize that NAAA inhibition will indirectly activate PPARα which in turn will reduce nicotine reward. The novel and selective NAAA inhibitor AM9053 [322] and AM11095, its analog with a better pharmacokinetic profile, were examined in the nicotine CPP test. AM9053 has been shown to potently inhibit NAAA (IC_{50}=30nM) and enhance OEA and PEA levels under naïve and inflammatory conditions [322,326]. NAAA inhibitors are typically used in pain-related studies, however the utilization of these compounds in nicotine reward may provide insight on the role of ethanolamides in nicotine dependence.

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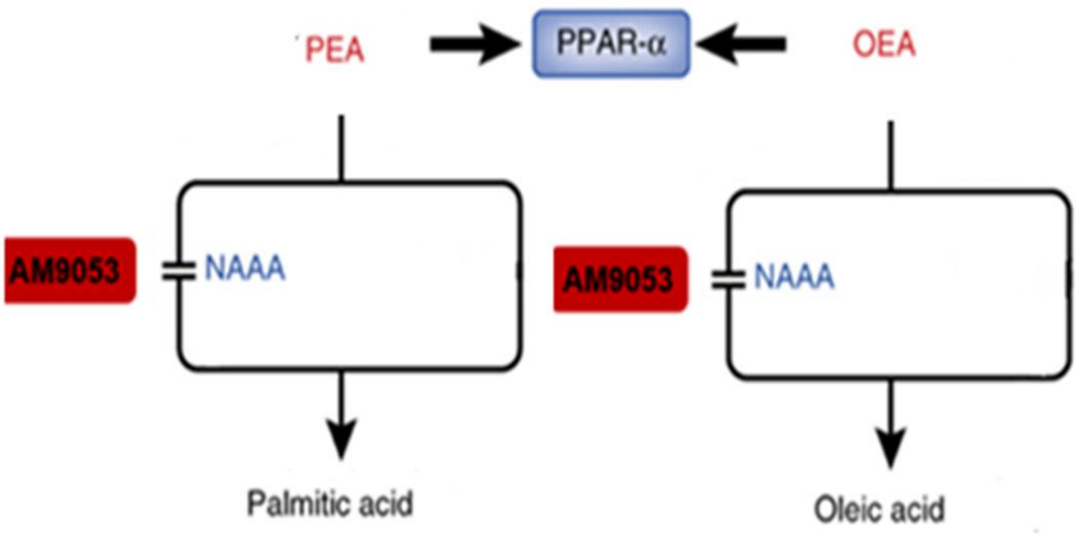

Figure 21: Schematic of Degradation of OEA and PEA (*Adapted from* [317])

The fatty acid ethanolamides OEA and PEA exert their effects primarily through PPARα. OEA and PEA are inactivated by the hydrolase NAAA into fatty acid and ethanolamine. The novel AM9053 compound selectively inhibits NAAA.

Figure 22: Structure of AM9053 (Structure provided by Dr. Alexandros Makriyannis' lab)

AM9053 inhibits NAAA activity with an IC_{50} value of 30nM. AM9053 showed a remarkable selectivity for human NAAA as compared to endocannabinoid serine hydrolase FAAH >100 uM [322].

B. Materials and Methods

Animals

Drug-naive, ICR male mice (8 weeks old upon arrival; Harlan Laboratories, Indianapolis, IN) served as subjects. Mice were housed four per cage with ad libitum access to food and water on a 12-h light cycle in a humidity and temperature controlled vivarium that was approved by the Association for Assessment and Accreditation of Laboratory Animal Care. Mice received corn cob bedding and were fed Envigo Teklad mouse/rat diet 7102 (LM-485). Experiments were performed during the light cycle and were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University and followed the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Drugs

(−)-Nicotine hydrogen tartrate [(−)-1-methyl-2-(3- pyridyl) pyrrolidine (+)-bitartrate] was purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). AM9053 and AM11095 were gifts from Dr. Alexandros Makriyannis of Northeastern University. AM9053 was dissolved in a mixture of 1:1:18 [1 volume ethanol/1 volume Emulphor-620 (Sanofi-Aventis, Bridgewater, NJ) and 18 volumes distilled water]. AM11095 was dissolved in a mixture of 1:1:18 [1 volume ethanol/1 volume Tween-80 (Sigma-Aldrich Co., St. Louis, MO) and 18 volumes distilled water] . Nicotine was injected s.c. and dissolved in saline. AM9053 and AM11095 were administered i.p. The nicotine solution pH was neutralized with sodium bicarbonate as needed. Freshly

prepared solutions were given to mice at 10 ml/kg. Doses are expressed as the free base of the drug.

Nicotine and Cocaine conditioned place preference studies

An unbiased CPP paradigm was performed. Briefly, the CPP apparatus consisted of three chambers in a linear arrangement (Med Associates, St Albans, VT). The CPP apparatus (MedAssociates, St. Albans, VT, ENV3013) consisted of white and black chambers (20×20×20 cm each), which differed in overall color and floor texture (white mesh or black rod). These chambers were separated by a smaller gray chamber with a smooth PVC floor. Partitions could be removed to allow access from the gray chamber to the black and white chambers. On day 1, animals were confined to the middle chamber for a 5-min habituation and then allowed to freely move between all three chambers for 15 min. Time spent in each chamber was recorded, and these data were used to populate groups of approximately equal bias in baseline chamber preference. Twenty-minute conditioning sessions occurred twice a day (days 2–4). During conditioning sessions, mice were confined to one of the larger chambers. The saline groups received saline in one large chamber in the morning and saline in the other large chamber in the afternoon. The drug group received drug in one large chamber and saline in the other large chamber. Treatments were counterbalanced equally in order to ensure that some mice received the unconditioned stimulus in the morning while others received it in the afternoon. The nicotine-paired chamber was randomized among all groups. Sessions were 4 h apart and were conducted by the same investigator. On each of the conditioning days, mice were pretreated with AM9053(i.p.), AM11095(i.p.) or its vehicle 2 hr or 1hr prior to nicotine injection respectively. On test day (day 5), mice were allowed access to all chambers for 15 min in a drug

free state. The preference score was calculated by determining the difference between the time spent in the drug paired side during test day versus the time in drug paired side during the baseline day.

Statistical analysis

Data were analyzed using the GraphPad software version 6.0 (GraphPad Software, Inc., La Jolla, CA) and expressed as the mean ± S.E.M. A one-way analysis of variance (ANOVA) in conjunction with Holm-Šídák comparison tests were conducted to determine significant effects of drug treatments vs controls. Comparisons were considered statistically significant when $p < 0.05$.

C. Results

Development of Nicotine CPP Attenuated by NAAA Inhibitor AM9053

Mice were conditioned with either saline or nicotine (0.5 mg/kg; s.c.) for 3 days in the CPP paradigm. In Fig. 23 a robust CPP was observed in nicotine–conditioned mice pre-treated with vehicle [$F(4, 30) = 7.990$, $p=0.0002$]. AM9053 given 2 hr prior to nicotine reduced nicotine reward. As revealed by the Holm-Šídák comparison tests, AM9053 (3mg/kg) significantly altered nicotine CPP ($p<0.05$), but was ineffective at the lower dose of 1 mg/kg ($p>0.05$). AM9053 at the highest dose used (3 mg/kg) did not produce a preference or aversion in saline treated-mice.

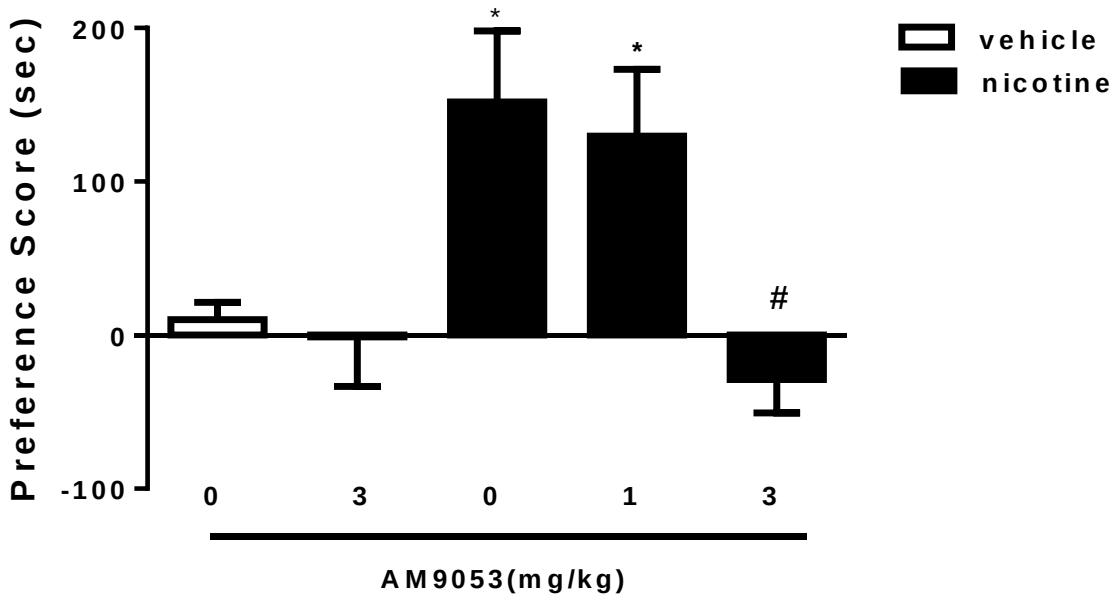

Figure 23. The Effect of NAAA Inhibitor AM9053 on Nicotine CPP.

Mice were conditioned with either s.c. saline or nicotine (0.5mg/kg) for 3 days. A robust CPP was observed in nicotine-conditioned mice pre-treated with vehicle. AM9053 (1 and 3mg/kg; i.p.) reduced nicotine reward as measured by the CPP test.*Denotes $p<0.05$ from vehicle control; # Denotes $p<0.05$ from nicotine control. Each point represents the mean ± SEM of n=6-8 mice per group.

Development of Nicotine CPP Attenuated by NAAA Inhibitor AM11095

Mice were conditioned with either saline or nicotine (0.5 mg/kg; s.c.) for 3 days in the CPP paradigm. In Fig. 24 a robust CPP was observed in nicotine–conditioned mice pre-treated with vehicle [$F(4, 32) = 6.490$, $p=0.0006$]. AM11095 given 1 hr prior to nicotine reduced nicotine reward. As revealed by the Holm-Šídák comparison tests, AM11095 (5mg/kg) significantly altered nicotine CPP ($p<0.05$), but was ineffective at the lower dose of 1mg/kg ($p>0.05$). AM11095 at the highest dose used (5 mg/kg) did not produce a preference or aversion in saline treated-mice.

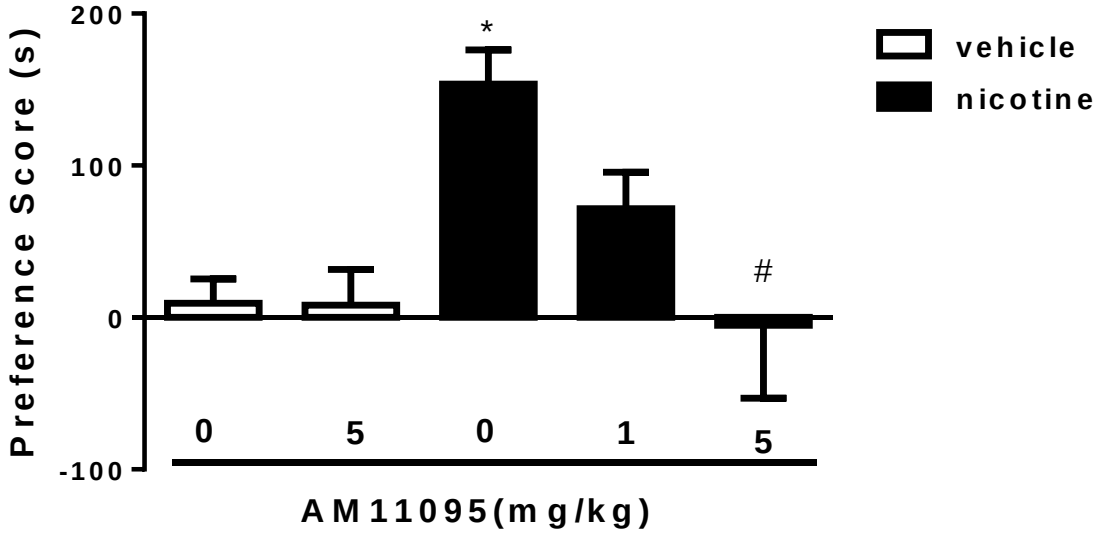

Figure 24. The Effect of NAAA Inhibitor AM11095 on Nicotine CPP

Mice were conditioned with either s.c. saline or nicotine (0.5mg/kg) for 3 days. A robust CPP was observed in nicotine-conditioned mice pre-treated with vehicle. AM11095 (1 and 5mg/kg; i.p.) reduced nicotine reward as measured by the CPP test. * Denotes $p<0.05$ from vehicle control; # Denotes $p<0.05$ from nicotine control. Each point represents the mean ± SEM of n=6-8 mice per group.

D. Discussion

The present study is the first to report the impact of the pharmacological inhibition of the lysosomal enzyme NAAA, degradative enzyme for OEA and PEA, in nicotine reward. Our results show that NAAA inhibition by AM9053 and AM11095 attenuates nicotine preference. AM9053 was shown to be highly selective and potent (IC_{50} = 30nM) *in vitro* for NAAA blockade and has efficacy in an *in vivo* murine model of colitis [322] and attenuated expression of inflammatory markers caused by lipopolysaccharide-induced macrophage activation[326]. AM9053 increases PEA and OEA levels after repeated administration *in vivo* or 8 hr incubation *in vitro* [322,326]. AM9053 enhances the levels of OEA and PEA in control J774 macrophage cells[326]. AM9053 has also been shown to increase the levels of other ethanolamides such as stearoylethanolamide, AEA, and docosahexaenoylethanolamide [326]; however, OEA was increased to a greater extent. After systemic administration, AM9053 not only enhanced PEA levels in the colon but the liver as well [322]. In addition, it has been shown that AM9053 does not enhance cerebellum PEA levels in mice with trinitrobenzene sulfonic acid-induced colitis[322]. The efficacy of AM11095 and its effect on ethanolamide levels is not available. There are reports that suggest NAAA inhibitors mediate anti-inflammatory and antinociceptive effects in animal models of pain and inflammation [322,323,327] through a PPARα-mediated mechanism[325,328]. Thus, our findings in nicotine CPP are consistent with the premise that NAAA inhibition indirectly activates PPARα. PPARα activation reduces nicotine reward and reinforcement[154,329] in rodents and nonhuman primates. In addition, it has been previously shown that methOEA (a long-lasting analog of OEA) reduces the rewarding effect of nicotine in the intravenous self-administration after systemic administration in rats [277]. In addition, OEA and PEA block nicotine-induced VTA

dopaminergic neuron excitation in a PPARα dependent manner [115]. Additional studies will further the understanding of the ethanolamide system in nicotine dependence

CHAPTER FIVE

GENERAL DISCUSSION

A. Rationale

Tobacco use is one of the leading causes of preventable deaths in the world[5]. There are current smoking cessation aids available; however, these therapies are modestly successful with less than 30% of users remaining abstinent for more than 1 year [15]. Therefore, there is a need for more efficacious therapies and this need may be met by a better understanding of the molecular underpinnings that induce nicotine dependence. Nicotine, the main addictive component in tobacco, exerts its effects through nAChRs [108]. One of the most abundant nAChRs in the brain, the homomeric α7 nAChR, has unique features and its role in nicotine dependence is not well understood. α7 nAChRs rapidly desensitize, have a low probability of being open [211] and high calcium permeability [50]. Preclinical data suggests that α7 nAChR activation attenuates nicotine reward [30,35] and pharmacological/genetic blockade of α7 nAChR enhances nicotine reward and reinforcement [30,35]. The characteristics of the α7 nAChR and its complex circuitry (see Ch.1 Section F and Fig. 1 for details) may account for these behavioral observations. Thus, the first aim of this dissertation was to investigate the impact of desensitization and channel opening of α7 nAChRs using pharmacological modulators such as PAMs and a silent agonist.

As previously mentioned, the α7 nAChR with its high calcium permeability induces signaling pathways that have been implicated in the areas of pain and cognition. With the interesting findings for the behavioral data, we sought to investigate a possible signaling cascade activated by α7 nAChR that may further elucidate its role in nicotine dependence. Therefore, the second

aim of this dissertation sought to elucidate a possible mechanism underlying the α7 nAChR by investigating PPARα as a downstream mediator of the α7 nAChR..

B. Summary of Results

Chapter 2 focused on aim 1 by elucidating the effects of α7 nAChR conformational changes in nicotine reward and withdrawal by utilizing pharmacological interventions. α7 nAChR orthosteric agonist PNU282987, Type I PAM NS1738, Type II PAM PNU120596, and the silent agonist NS6740 were used. The α7 full orthosteric agonist PNU282987 and the Type II α7 nAChR PAM PNU120596 reduced nicotine CPP (Fig. 4 and 6) while the silent agonist NS6740 and Type I PAM NS1738 had no effect (Fig. 5 and 7). In nicotine withdrawal, PNU282987, NS1738, and PNU120596 attenuated different aspects of the withdrawal syndrome (Fig.8, 9 and 10). In the nicotine withdrawal experiments the orthosteric agonist PNU282987 attenuated anxiety-like behaviors (Fig.8); however, the α7 nAChR PAMs NS1738 and PNU120596 had no effect on anxiety-like behavior as observed in the elevated plus maze (Fig. 9 and 10). The orthosteric full agonist PNU282987 and they Type I PAM NS1738 both attenuated somatic signs, but the Type II PAM PNU120596 had no effect on somatic signs. PNU120596 was the only ligand to reduce hyperalgesia. To our knowledge, this is the first report of α7 nAChR PAMs and a silent agonist used in preclinical nicotine dependence tests. The results from chapter 2 highlighted the importance of α7 nAChR desensitization, probability of channel opening, and endogenous tone.

The next chapter (Chapter 3) focused on aim 2 and investigated a potential mediator of the α7 nAChR, PPARα. This chapter suggests that α7 nAChR activation attenuates nicotine CPP in a PPARα-dependent mechanism (Fig.12). In addition, the PPARα agonists WY-14643 and fenofibrate attenuated nicotine preference as expected but fenofibrate was less effective and not

PPARα-dependent (Fig. 13, Fig.17 and Fig.18). In addition, in contrast to WY-14643, fenofibrate had a modest efficacy in reducing nicotine withdrawal signs (Fig.19 and Fig.20). Chapter 4 is a continuation of the theme of Chapter 3, but with an emphasis on indirect activation of PPARα. This is a short chapter on the inhibition of NAAA, the degradative enzyme for OEA and PEA, in nicotine reward. The results show that NAAA inhibition attenuates nicotine preference in mice (Fig.23 and Fig.24), which is consistent with the premise that NAAA inhibition indirectly activates PPARα.

C. Discussion of Results

Collectively, these results suggest that the role α7 nAChR in nicotine dependence is conformation-dependent and mediated by PPARα. The finding in Chapter 2 that the silent agonist NS6740 has no effect on nicotine CPP (Fig.7) is similar to its lack of effect in a preclinical model of cognitive function[245]. This suggests ion conductance/receptor activation is necessary for the α7 nAChR induced reduction of nicotine CPP. This result also supports the role of PPARα mediation in the effect of the α7 nAChR, as suggested in Chapter 3. Indeed, α7 nAChR pharmacological activation by PNU282987 enhanced the neuronal levels of endogenous PPARα ligands OEA and PEA in the VTA in a Ca^{2+}-dependent manner [116]. However, if the notion of the necessity of channel activation is valid, it is unclear why the α7 nAChR Type I PAM NS1738 (1 and 10mg/kg) was ineffective at reducing nicotine CPP. NS1738 increases the probability of opening of α7 nAChRs. The increase in channel opening would increase the likelihood of ion conductance, thus it is plausible that NS1738 would be more effective than the orthosteric agonist PNU282987 at attenuating nicotine CPP. Higher doses of NS1738 than those used in our current study have been effective in inflammation studies[240] and may also induce an effect in nicotine CPP.

In addition, this dissertation is the first to utilize α7 nAChR PAMs in nicotine CPP and withdrawal. The results suggests the presence of an endogenous tone mediated through α7 nAChRs that modulates nicotine reward and withdrawal. The Type I PAM NS1738 attenuated nicotine withdrawal-induced somatic signs (Fig. 9). The Type II PAM PNU120596 attenuated nicotine CPP (Fig. 6) and nicotine withdrawal-induced hyperalgesia (Fig. 10). The modulation of the endogenous tone is receptor conformation dependent. In particular, these findings may suggest that individuals with low endogenous α7 nAChR activation are more likely to develop nicotine dependence. This dissertation adds to the understanding of the endogenous cholinergic system in nicotine dependence.

The neurotransmitter systems of the brain such as glutamate, GABA, dopamine, and acetylcholine have been implicated in aspects of nicotine dependence. Nicotine targets nAChRs and induces its dependency effects. The β2* nAChRs are required for nicotine reward, reinforcement and some aspects of withdrawal [93,135,183]. Nicotine has a low affinity for the α7 nAChR and initial preclinical studies suggested that this receptor was not involved in the rewarding effects induced by nicotine [37]. However, recently it has been shown that the α7 nAChR modulates nicotine reward [30,35]. This may be due to its neurophysiological modulation of neurotransmitter systems involved in nicotine dependence. The locality of α7 nAChRs on presynaptic terminals and postsynaptically allow this receptor to modulate neurotransmitter release and participate in fast synaptic transmission. The circuitry of the α7 nAChR in the mesolimbic system provides multiple possible pathways the α7 nAChR can modulate dopamine release (Fig. 1). For instance, the preterminal α7 nAChRs on glutamatergic afferents in the NAc potentiate glutamate release and are synapsed on to medium spiny neurons. Activation of these α7 nAChRs can indirectly activate ionotropic glutamate receptors on dopaminergic axon

terminals [221,222] which results in dopamine release. However, if preterminal α7 nAChRs were desensitized the net outcome would be a reduction of dopamine release. Another potential outcome of preterminal α7 nAChR activation on glutamatergic axon terminals in the NAc is attenuation of dopamine release via activation of metabotropic glutamate receptor activation. [223]. This outcome will result in enhancement of dopamine release if α7 nAChRs are desensitized. The results from Ch.2 of this dissertation may provide a behavioral understanding of α7 nAChRs in nicotine dependence, but it does not aid in determining which pathways are activated or desensitized. The attenuation of nicotine CPP by the Type II PAM PNU120596, which increases the probability of channel opening and blocks desensitization, may suggest that through delayed desensitization or resensitization of an α7 nAChR-mediated inhibitory pathway on dopamine was activated. In addition, the effect of PNU120596 is dependent on the endogenous acetylcholine/choline tone. Thus, enough endogenous tone was provided for PNU120596 to induce an effect. The lack of effect of the silent agonist NS6740 may suggests that this ligand desensitized an inhibitory α7 nAChR pathway. Further molecular and behavioral studies may elucidate the role of α7 nAChR circuitry in nicotine dependence.

The PPARα and α7 nAChR interaction may occur at postsynaptical α7 nAChRs in the VTA on dopaminergic neurons [218,330]. PPARα is a nuclear hormone receptor that is predominately found in the nucleus or the surrounding cytoplasmic space [295,331,332]. Furthermore, the ethanolamides OEA, PEA, and the endocannabinoid AEA are made on demand and are thought to be synthesized by a membrane bound enzyme [320]. AEA is released postsynaptically to engage in retrograde transmission [333] thus, it is reasonable to believe that AEA is synthesized in the soma along with other ethanolamides such as OEA and PEA. Interestingly, it has been suggested that mice lacking the α7 nAChR showed a steady increase in nicotine induced dopamine outflow

over time in the nucleus accumbens which was in contrast to WT mice [256]. This may suggest that α7 nAChRs serve as an inhibitory regulator of dopamine release in the VTA. It has been previously postulated that α7 nAChRs may modulate β2* nAChR-induced dopamine release via PPARα in the VTA[116,278]. Indeed, nicotine-induced dopamine release is β2* nAChR dependent[135]. Further physiological and behavioral studies are needed to understand this interaction.

D. Future Directions

The overall future directions of this dissertation are to elucidate the neurocircuitry and pharmacology of α7 nAChRs and PPARα in nicotine CPP and withdrawal, in hopes to implicate these receptors as viable targets for smoking cessation aids. The pharmacological ligands used in this dissertation were systemically administered; therefore, local infusions of the pharmacological ligands administered in nicotine CPP and nicotine withdrawal will aid in determining the neurocircuitry involved. NAAA, PPARα, and α7 nAChRs are expressed in brain regions associated with reward such as the prefrontal cortex, NAc and VTA [41,122,289,321,334,335]. Also, these regions are involved in nicotine CPP [36,139,336]. Genetically modified mice such as floxed α7 nAChR mice[337] may provide an understanding of the neural substrates involved. Even though CPP and self-administration were originally thought to be isomorphic models for measuring drug reward, it is now accepted that CPP measures drug reward and self-administration measures drug reinforcement [71]. Thus to further extend the understanding of our finding in nicotine dependence, it is important to test the mechanisms in this dissertation in nicotine intravenous self-administration and reinstatement. Furthermore, SPPARMS for PPARα such as K-877[309] may have a higher potency than original fibrate compounds because they

interact with the large binding pocket of PPARα to induce a different co-factor recruitment[310]. Therefore, it is important to test SPPARMS in nicotine dependence assays.

In addition, we will continue to characterize the NAAA inhibitors in nicotine CPP by investigating the PPARα mediation of its effects. OEA and PEA have other targets such as G-protein-coupled receptor 55, transient receptor potential cation channel subfamily V member, and G-protein-coupled receptor 119 [338–340]. Administering OEA and PEA systemically in nicotine dependence assays will further implicate ethanolamides in nicotine dependence. Also, the effect of NAAA inhibition in nicotine withdrawal will provide more evidence of the involvement of the ethanolamide system in nicotine dependence.

There is a dire need for new molecular targets for smoking cessation therapies. The β2* nAChRs have been extensively studied and are the targets for some of the current therapies. However, given the modest efficacy of the current smoking cessation aids, it suggest the need for new molecular targets. This dissertation focused on the α7 nAChR and PPARα as potential new targets for smoking cessation aids. Our work and others suggest that the α7 nAChR may act as a molecular break that attenuates nicotine rewarding effects produced by high affinity nAChR subtypes. Therefore, selectively activating α7 nAChRs may reduce the rewarding effects of nicotine even in individuals who are currently using tobacco products. α7 nAChR agonists and modulators are undergoing clinical trials to enhance cognitive function, thus, these ligands can be repurposed as smoking cessation aids [341,342]. In addition, this dissertation suggests that PPARα mediates the attenuation of α7 nAChRs in nicotine CPP. Also, activation of this receptor has been previously shown to attenuate nicotine reward and reinforcement. Furthermore, this dissertation is the first to report PPARα activation attenuates nicotine withdrawal signs. The K-877 SPPARM for PPARα is undergoing clinical trials[309] and can also be repurposed as a

smoking cessation aid. Taken together the results from this dissertation aids support the development of α7 nAChR agonists and more potent PPARα activators such as K-877 as possible smoking cessation aids.

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VITA

Asti Bre'un Jackson was born on January 29, 1991 in Mobile, Alabama. Her family moved to Georgia when she was five and resided in cities within Dekalb County. Asti attended schools in Lithonia, Ga. Her teacher Ms. Lockhart sparked her interest for science in her 7th grade Life Science class at Salem Middle School. Ms. Lockhart enthusiastically taught about Punnett squares and recessive traits and the topic caught Asti's attention. It was during that period Asti was told she herself had sickle cell trait and she had a half-brother with sickle cell disease. From that point on Asti was interested in becoming a genetic counselor. Asti graduated from Martin Luther King Jr. High School in 2009.

In 2009, she began her college career entering Georgia State University in Atlanta, GA. Asti majored in biology and was focused on becoming a genetic counselor until she met the director of the Ronald E. McNair Postbaccalaureate Achievement Program. This program aims to increase the number of underrepresented minorities entering into graduate school. The director encouraged her to apply and to pursue a Ph.D. In 2012 she was accepted into the Ronald E. McNair Postbaccalaureate Achievement Program and conducted research on cocaine addiction in Dr. Kyle Frantz's lab. In 2013, she graduated Magna cum laude with a BS in Biology from Georgia State University.

Asti then enrolled in Fall of 2013 at Virginia Commonwealth University in the Biomedical Sciences Doctoral Portal Program. In 2014, Asti joined Dr. M. Imad Damaj's lab in the Pharmacology and Toxicology Department and conducted nicotine dependence research.

Asti Bre'un Jackson

Education

Virginia Commonwealth University, Richmond, VA 2013-Present
Doctorate of Philosophy in *Pharmacology* and *Toxicology*
• Travel Award Recipient for Chemistry and Pharmacology of Drugs of Abuse Conference, 2016

Georgia State University, Atlanta, GA 2009-2013
Bachelor of Science in *Biology*, Magna cum laude
• Georgia's HOPE Scholarship, 2009-2013
• International Education Fee Study Abroad Scholarship, 2012
• Ronald E McNair Scholar, 2012-Present

Research Experience

Doctoral Dissertation, Virginia Commonwealth University, Richmond, VA 2013-Present
Adviser: Dr. M. Imad Damaj
Investigating the Modulations and Mechanisms of Alpha Nicotinic Acetylcholine Receptors in Nicotine Dependence
• Investigated the effect of physiological characteristics of alpha 7 nicotinic acetylcholine receptors using pharmacological interventions and implicated the peroxisome proliferator activated receptor alpha as a downstream mediator of the alpha 7 nicotinic acetylcholine receptor.

Undergraduate Ronald E. McNair Scholar, Georgia State University, Atlanta, GA 2012-2013
Adviser: Dr. Kyle Frantz
Impact of Cocaine Self-Administration in Adolescent vs Adult Rats
• Investigated the effect of white noise on lever pressing behaviors in rats

Teaching Experience

Teaching Assistant, Department of Chemistry**,** Georgia State University, Fall 2011
• Provided support for faculty member and guided students with cobalt synthesis experiments.

Guest Lecturer, Drug Biology 491 Course, Virginia Commonwealth University Nov. 2015,2016
• Taught undergraduate students about nicotine dependence and made test questions

Leadership Experience

President, Virginia Commonwealth University Pharmacology and Toxicology Student Organization 2015-2016.
Public Relations Chair, Black Graduate Student Association, Virginia Commonwealth University 2016-Present
Mentor, Professional and Personal Development Class 2016
Mentor, Big Brothers and Big Sisters Organization, 2015-2016
Secretary, Social Justice Ministry, Sixth Mount Zion Baptist Church, 2016-2017
Student Representative, Virginia Commonwealth University, Department Retreat Committee 2016
Conference Manager, Georgia State University Housing, 2013
Resident Assistant, Georgia State University Housing, 2012-2013
Secretary, Georgia State University, Beta Beta Beta Biological Honor Society 2012-2013

Research Publications

Jackson A, Bagdas D, Muldoon P ,Lichtman A, Carroll FI, Greenwald M, Miles M, and Damaj MI (2017) In vivo Interactions between α7 Nicotinic Acetylcholine Receptor and Nuclear Peroxisome Proliferator-Activated Receptor- α: Implication for Nicotine Dependence. Neuropharmacology 118:38-45

Slater C., **Jackson A**.*, Muldoon P., Dawson A., O'Brien M., Soll L., Abdullah R., Carroll FI, Tapper A., Miles M., Banks M, Damaj MI (2016) Nicotine Enhances the Hypnotic and Hypothermic Effects of Alcohol in the Mouse. Alcohol: Clinical and Experimental Research 40(1):62-72 ***co-first author**

Alsharari SD, King JR, Nordman JC, Muldoon PP, **Jackson A**, Zhu AZ, Tyndale RF, Kabbani N, Damaj MI. (2015) Effects of Menthol on Nicotine Pharmacokinetic, Pharmacology, and Dependence in Mice. PLoS One 10(9):e0137070

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Enga R, M, **Jackson A**, Damaj MI, Beardsley PM (2016) Oxycodone physical dependence and its oral self-administration in C57BL/6J mice. European Journal of Pharmacology 789:75-80

Jackson KJ, **Jackson A**, Ivy Carroll F, Damaj MI (2015) Effects of orally-bioavailable short-acting kappa opioid receptor-selective antagonist LY2456302 on nicotine withdrawal in mice. Neuropharmacology 97:270-4

Professional Oral Presentations

Jackson A., and Damaj M. (2016) Investigating the Role of Peroxisome Proliferator-Activated Receptor Type-α in Nicotine Dependence. Carolina Cannabinoid Collaborative Meeting in Philadelphia, PA

Jackson A., and Damaj M. (2014) Investigating the Genetics of Nicotine Dependence Using Mouse Models. Virginia Commonwealth University Biomedical Sciences Doctoral Portal in Richmond, VA

Professional Poster Presentations

Jackson A., Bagdas D., Muldoon P., Lichtman A., Carroll FI, Miles M. and Damaj M. (2016). Investigating the Role of the α7 Nicotinic Acetylcholine Receptors in Nicotine Dependence. Society for Neuroscience in San Diego, CA

Jackson A., Muldoon P., Damaj M. (2016) The Role of the α7 Nicotinic Acetylcholine Receptor in Nicotine Dependence. Chemistry and Pharmacology of Drugs of Abuse Conference in Boston, MA

Jackson A., Bagdas D., Damaj M. (2016) Investigating the Role of the α4β2 Nicotinic Receptor Positive Allosteric Modulator Desformylflustrabromine in Nicotine Dependence. Virginia Brain Rx Symposium in Richmond, VA

Jackson A., Muldoon P., Damaj M. (2015) Nicotine Reward Modulated by α7 Nicotinic Acetylcholine Receptor and Peroxisome Proliferator-Activated Receptor α Interaction. Mid-Atlantic PREP/IMSD Research Symposium in Raleigh, NC

Jackson A., Alsharari S., Siu E., Tyndale R., Kabbani N., Damaj M. (2015) Effects of Menthol on Nicotine Pharmacokinetic, Pharmacology, and Dependence in Mice. Society for Research on Nicotine and Tobacco Meeting in Philadelphia, PA

Jackson A., Muldoon P., Damaj M. (2014) Peroxisome Proliferator-Activated Receptor Type-α Agonists as New Treatments for Nicotine Dependence. Carolina Cannabinoid Collaborative Meeting in Winston-Salem, NC

Jackson A., Slater C., Muldoon P., Damaj M., (2013) Acute and Chronic Nicotine-Ethanol Interaction in the Loss of Righting Reflex Test. Research Colloquium at Virginia Commonwealth University in Richmond, VA

Jackson A., Polites J., Williams B., Frantz K., (2012) Comparison of Locomotor Activity in Adolescent and Adult Male Rats during Cocaine Self-Administration. Annual Biomedical Research Conference for Minority Students Conference in San Jose, CA