



# VCU

Virginia Commonwealth University  
VCU Scholars Compass

---

Theses and Dissertations

Graduate School

---

2012

## THE ANTINOCICEPTIVE EFFECTS OF ALPHA 7 NICOTINIC ACETYLCHOLINE RECEPTOR POSITIVE ALLOSTERIC MODULATORS IN DIFFERENT ANIMAL PAIN MODELS

Kelen Freitas  
*Virginia Commonwealth University*

Follow this and additional works at: <https://scholarscompass.vcu.edu/etd>



Part of the [Medical Pharmacology Commons](#)

© The Author

---

Downloaded from

<https://scholarscompass.vcu.edu/etd/2827>

This Thesis is brought to you for free and open access by the Graduate School at VCU Scholars Compass. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of VCU Scholars Compass. For more information, please contact [libcompass@vcu.edu](mailto:libcompass@vcu.edu).

© Kelen Cristina Cardoso de Freitas 2012

All Rights Reserved

THE ANTINOCICEPTIVE EFFECTS OF ALPHA 7 NICOTINIC ACETYLCHOLINE  
RECEPTOR POSITIVE ALLOSTERIC MODULATORS IN DIFFERENT ANIMAL PAIN  
MODELS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science  
at Virginia Commonwealth University.

by

KELEN CRISTINA CARDOSO DE FREITAS

Bachelor of Science, Pharmacy, CESUMAR, Centro Universitario de Maringá, 2006  
Post-Bachelor, Pharmaceutical Manipulation, Centro Universitário de Maringá, 2008

Director: M. Imad Damaj, PhD

Professor, Department of Pharmacology and Toxicology



Virginia Commonwealth University

Richmond, Virginia

May 2012

## Acknowledgements

“ The will of God will never take us where the Grace of God cannot sustain us.” (Billy Graham). Father God, I know that you see the desires of my heart. Today I bring them before you and resolve to continue doing so, knowing that you are Good. Thank you for this Grace. I would like to offer my most heartfelt appreciation and gratitude to my mother, who supported me throughout my graduate school career. My mother left Brazil when I was 14 years old and came to the United States to start a new life. One of the main reasons that she came to the United States was to give my brother and I a better education. She is the best example I know of someone that is very determined, focused, goal-oriented and incredibly hard-working. She is an example of pure, unconditional love. She has never asked for anything but that I follow my heart. Thank you so much mom. I would like also to thank my father and brother for their love, enthusiasm, and encouragement throughout my studies. I also extend a special thanks to my stepfather for being my sponsor and principal enabler for my time in the U.S.A.

Though I initially wanted to describe my advisor’s role in my project as unique. I realized that Dr. Damaj’s help truthfully constitutes precisely what it means to be an advisor and mentor, and thus unique was not the proper term. The extent to which Dr. Damaj filled his role in my project can only be described as unimaginable. His patience and guidance at every stage of my work was remarkable, while his understanding and friendship provided a support for which I am immeasurably grateful. Only he knows, as I do, the extent of the work that went into writing the submitted peer-reviewed articles and this thesis, and through his unwavering dedication to respond to my sometimes hourly emails, among other hardships that I am sure I put him through. I have gained a multitude of skills that I would not have otherwise dreamed of gaining. I truthfully could not have imagined a better advisor and mentor for my Master’s degree work, and thus, Dr. Damaj, I thank you.

Besides my advisor, I would like to gratefully acknowledge my thesis committee. Dr. Guy Cabral, Dr. Lichtman and Dr. Negus, for their encouragement, insightful comments, advice and crucial contribution in this project. I am thankful that in the midst of all their activity, they accepted to be members of the reading committee. Of note, I thank Dr. Negus for his valuable advice and immense help and furthermore, using his precious times to work with me on my isobolographic analysis. I really appreciate it.

I would also like to thank all of my labmates, who created a convivial place to work. In particular, I would like to thank Shakir for being a great mentor to me, especially at the start of my project. I thank my coworker Tie Han for his instruction, guidance, and support throughout this entire process. I also thank Pretal and Cindy for their instruction and friendship when I joined the lab. I thank Mai, for her motivation and encouragement during difficult, troublesome, and even joyous times. All other labmates, Sarah, Anton and Ali had inspired me in research and life through our interactions during the long hours in the lab. I also thank Dr. Kia Jakson for her constructive comments on my research. I have worked with great number of people whose contribution in assorted ways to the research and making of the thesis deserved special mention. I’d like to thank my best friend Ashok for making biochemistry class fun and also relieving my stress and always finding a way to make me smile.

## Table of Contents

	Page
Acknowledgements .....	ii
List of Figures .....	xi
List of Tables .....	xiv
List of Abbreviations .....	xv
Abstract .....	xvi
Chapter I	
1.1 Review of the Literature.....	1
1.2 Pain Modalities.....	2
1.3 Animal Models of Pain and Inflammation .....	3
1.3.1 Acute Thermal Pain Tests.....	4
1.3.1.1 Hot-Plate Test.....	4
1.3.1.2 Tail-Flick Test .....	5
1.3.2 Acute Mechanical Pain Test.....	5
1.3.3 Persistent Pain Model.....	6
1.3.4 Inflammatory Pain Model .....	6
1.3.5 Neuropathic Pain Model.....	7

1.4 Available Treatments and Challenges .....	8
1.5 Nicotinic Acetylcholine Receptors .....	9
1.5.1 Subunit Composition, Stoichiometry and Distribution .....	10
1.5.2 Nicotinic Receptors in Nociceptive Systems .....	11
1.5.3 $\alpha 7$ Nicotinic Receptors in Pain Pathways.....	13
1.5.4 $\alpha 7$ Nicotinic Receptors in the Cholinergic Anti-inflammatory System...	15
1.6 Positive and Negative Allosteric Modulation of nAChRs.....	17
1.6.1 Advantages of Using Positive Allosteric Modulators .....	19
1.7 Allosteric Modulation of $\alpha 7$ nAChRs .....	20
1.8 Thesis Objectives .....	24
<b>Chapter II The Antinociceptive Effects of Nicotinic Receptors Alpha 7 Positive Allosteric Modulators in Murine Acute and Tonic Pain Models</b>	
2.1 Chapter II Introduction .....	26
2.2 Materials and Methods .....	27
2.2.1 Animals .....	27
2.2.2 Drugs .....	28
2.2.3 Antinociceptive Tests.....	28
2.2.3.1 Tail-Flick Test .....	28

2.2.3.2 Hot-Plate Test.....	29
2.2.3.3 Mechanical Sensitivity Test .....	30
2.2.3.4 Formalin Test.....	30
2.2.4 Intrathecal Injections .....	31
2.2.5 Locomotor Activity Test .....	31
2.2.6 Motor Coordination Test .....	32
2.2.7 Chronic PNU-120596 Administration Protocol .....	32
2.2.8 Time Course of PNU-120596 and NS1738 levels in Brain and Plasma ..	32
2.2.9 Statistical Analysis.....	33
2.3 Results .....	33
2.3.1 Lack of Antinociceptive Effect of $\alpha 7$ PAMs in Acute Thermal Pain in Mice .....	33
2.3.2 The Effects of $\alpha 7$ PAMs on Mechanical Sensitivity .....	36
2.3.3 Time Course of Type I $\alpha 7$ PAMs in the Formalin Test.....	38
2.3.4 Dose-Response Curve of Type I $\alpha 7$ PAM in the Formalin Test .....	40
2.3.5 Time Course of Type II $\alpha 7$ PAM in the Formalin Test .....	42
2.3.6 Dose-Response Curve of Type II $\alpha 7$ PAM in the Formalin Test .....	44

2.3.7 Effects of Type I and II $\alpha 7$ PAMs after i.t. Administration in the Formalin Test.....	46
2.3.8 NS1738 Blocks PNU-120596's Antinociceptive Effect in the Formalin Test .....	48
2.3.9 The Lack of Effect of $\alpha 7$ PAM on Locomotor Activity and Coordination of Mice .....	50
2.3.10 Time Course of a Selective $\alpha 7$ Agonist in the Formalin Test.....	52
2.3.11 Dose-Response Curve of a Selective $\alpha 7$ Agonist in the Formalin Test .....	54
2.3.12 Role of $\alpha 7$ and $\beta 2^*$ nAChRs Subtypes in PNU-120596-induced Antinociception in the Formalin Test.....	56
2.3.13 Role of $\alpha 7$ nAChRs Subtypes in PNU-120596-induced Antinociception in the Formalin Test .....	58
2.3.14 Role of $\mu$ opioid Receptors in PNU-120596-induced Antinociception in the Formalin Test .....	60
2.3.15 Contribution of Peripheral and Spinal $\alpha 7$ nAChRs to PNU-120596's Antinociceptive Response.....	62
2.3.16 The Effects of i.t. Administration of the MEK inhibitor U-0126 on PNU-120596's Antinociceptive Effect in the formalin Test .....	64



2.3.17 Tolerance did not Develop to PNU-120596's Effect in the Formalin Test after Chronic Exposure .....	66
2.3.18 Measurement of $\alpha 7$ Type I and II PAMs Levels in the Brain and Plasma .....	68
2.4 Summary of Chapter II Results .....	72
Chapter III In Vivo Pharmacological Interactions Between Type II Positive Allosteric Modulators of $\alpha 7$ Nicotinic Acetylcholine Receptors and Nicotinic Agonists in Mice	
3.1 Chapter III Introduction .....	74
3.2 Materials and Methods .....	75
3.2.1 Animals .....	75
3.2.1 Drugs .....	75
3.2.3 Antinociceptive Tests .....	76
3.2.3.1 Drug Interactions in the Formalin Test .....	76
3.2.3.2 Tail-Flick Test .....	77
3.2.3.3 Hot-Plate Test .....	77
3.2.4 Body Temperature Measure .....	77
3.2.5 Locomotor Activity Test .....	78

3.2.6 Seizure Testing .....	78
3.2.7 Intrathecal Injections .....	78
3.2.8 Statistical Analysis .....	79
3.3 Results .....	80
3.3.1 Dose-response Analysis of PNU-120596 and Choline Alone and Their Combinations in the Formalin Test .....	80
3.3.2 Dose-response Analysis of PNU-120596 and Choline Alone and Their Combinations in the Formalin Test .....	83
3.3.3 PNU-120596 Enhances PHA-543613's effects in the Formalin Test .....	87
3.3.4 PNU-120596 Enhances Nicotine's Effects in the Formalin Test .....	89
3.3.5 PNU-120596 Failed to Enhance Morphine's Effects in the formalin Test	91
3.3.6 The Effects of PNU-120596 on Nicotine-induced Antinociception .....	93
3.3.7 The Effects of PNU-120596 on Nicotine-decrease in Locomotion, Seizures and Hypothermia .....	95
3.3.8 Characterization of Nicotine-induced Hypothermia in the Presence of PNU- 120596 .....	99
3.4 Summary of Chapter III Results .....	102

Chapter IV Effects Alpha 7 Positive Modulators in Murine Inflammatory and Chronic  
Neuropathic Pain Models

4.1 Chapter IV Introduction .....	103
4.2 Materials and Methods .....	104
4.2.1 Animals .....	104
4.2.2 Drugs .....	105
4.2.3 Intrathecal Injections .....	105
4.2.4 Prolonged Pain Models .....	105
4.2.4.1 Carrageenan Model of Short Inflammatory Pain .....	105
4.2.4.2 Chronic Constriction Injury (CCI) .....	107
4.2.5 Locomotor Activity .....	109
4.2.6 Motor Coordination .....	109
4.2.7 Statistical Analysis .....	110
4.3 Results .....	110
4.3.1 Effects of NS1738 and PNU-120596 on Developing Inflammation after Carrageenan Injection .....	110
4.3.2 Effect of PNU-120596 on Reversing Carrageenan-induced Thermal Hyperalgesia and Paw Edema .....	116
4.3.3 Effect of NS1738 on Thermal Hyperalgesia in the CCI Model .....	118

4.3.4 Effects of PNU-120596 on Thermal Hyperalgesia in the CCI Model ...	120
4.3.5 Effects of NS1738 and PNU-120596 on Mechanical Allodynia in CCI Mice .....	123
4.3.6 Effects of the Interaction Between PNU-120596 and PHA-543613 on Mechanical Allodynia in CCI Mice .....	125
4.3.7 Effects of NS1738 and PNU-120596 on Locomotor Activity of Mice ..	127
4.4. Summary of Chapter IV Results .....	129
Chapter V	
5.1 General Discussion .....	130
5.2 Significance of Findings .....	143
5.3 Future Directions .....	146
References .....	148

<b>List of Figures</b>	<b>Page</b>
Figure 1 .....	35
Figure 2 .....	37
Figure 3 .....	39
Figure 4 .....	41
Figure 5 .....	43
Figure 6 .....	45
Figure 7 .....	47
Figure 8 .....	49
Figure 9 .....	51
Figure 10 .....	53
Figure 11 .....	55
Figure 12 .....	57
Figure 13 .....	59
Figure 14 .....	61
Figure 15 .....	63

Figure 16 .....	65
Figure 17 .....	67
Figure 18 .....	71
Figure 19 .....	81
Figure 20 .....	85
Figure 21 .....	86
Figure 22 .....	88
Figure 23 .....	90
Figure 24 .....	92
Figure 25 .....	94
Figure 26 .....	97
Figure 27 .....	101
Figure 28 .....	113
Figure 29 .....	115
Figure 30 .....	117
Figure 31 .....	119
Figure 32 .....	122

Figure 33 .....	124
Figure 34 .....	126
Figure 35 .....	128

<b>List of Tables</b>	<b>Page</b>
Table 1 .....	82
Table 2 .....	98



## List of Abbreviations

TNF- $\alpha$	Tumor necrosis factor- $\alpha$
LPS	Lipopolysaccharides
CFA	Complete Freund's Adjuvant
PSL	Partial sciatic nerve ligation
SNL	Spinal nerve ligation
CCI	Chronic constriction injury
NSAIDs	non-steroidal anti-inflammatory drugs
nAChRs	Nicotinic acetylcholine receptors
CNS	Central nervous system
PNS	Peripheral nervous system
GABA	Glutamate and $\gamma$ -aminobutyric acid
ACh	Acetylcholine
i.t.	Intrathecal
MLA	Methyllycaconitine
DRG	Dorsal root ganglia
PAMs	Positive allosteric modulators
NAMs	Negative allosteric modulators
i.v.	Intravenously
i.c.v.	Intracerebroventricularly
5HT <sub>3</sub>	5-hydroxytryptamine
TMD	Transmembrane domain
CSF	Cerebrospinal fluid
BTX	[125]- $\alpha$ -bungarotoxin
ERK	Extracellular signal-regulated kinase
AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
DH $\beta$ E	Dihydro- $\beta$ -erythroidine
i.p.	Intraperitoneal
%MPE	Percent maximum possible effect
i.pl.	Intraplantar
BBB	Blood-brain barrier
s.c.	Subcutaneously
$\Delta$ PWL	Change in paw withdrawal latencies
NF- $\kappa$ B	Nuclear factor $\kappa$ B
cAMP	Adenosine monophosphate cyclic
pCREB	Response element binding protein
PKC	Protein kinase C

THE ANTINOCICEPTIVE EFFECTS OF ALPHA 7 NICOTINIC ACETYLCHOLINE  
RECEPTOR POSITIVE ALLOSTERIC MODULATORS IN DIFFERENT ANIMAL PAIN  
MODELS

By Kelen Cristina Cardoso de Freitas

A thesis submitted in partial fulfillment of the requirements for the degree of M.S. at Virginia  
Commonwealth University.

Director: M. Imad Damaj , Ph.D.  
Professor with the Department of Pharmacology and Toxicology

The  $\alpha 7$  nicotinic acetylcholine receptor (nAChR) subtype is abundantly expressed in the central nervous system (CNS) and in the periphery. Positive allosteric modulators (PAMs) of the  $\alpha 7$  increase the response to an agonist and are divided into two types depending on whether they also decrease desensitization of the receptor (type II) or not (type I). Therefore, this study aims to investigate whether the enhancement of endogenous  $\alpha 7$  nAChR function will result in a beneficial effect in nociceptive, inflammatory and chronic neuropathic pain models. While NS1738 and PNU-120596 were not active to reduce acute thermal pain, measured by hot-plate and tail-flick tests, only PNU-120596 dose-dependently attenuated paw-licking behavior in the formalin test. Our results with selective (MEK) inhibitor U0126 argues for an important role of extracellular signal-regulated kinase (ERK1/2) pathways activation in PNU-120596's antinociceptive effects in formalin-induced pain. The  $\alpha 7$  antagonist MLA, via intrathecal and intraplantar administration, reversed PNU-120596's effects, confirming PNU-120596's action through central and peripheral  $\alpha 7$  nAChRs. Tolerance to PNU-120596 was not developed after chronic treatment of the drug. Furthermore, mixtures of PNU-120596 and choline, an endogenous  $\alpha 7$  nAChR agonist, synergistically reduced formalin-induced pain, while interactions of non-antinociceptive doses of PNU-120596 and PHA-543613, a selective  $\alpha 7$  nAChR agonist, or nicotine resulted in antinociception. In contrast, PNU-120596 failed to enhance nicotine-induced convulsions, -hypomotility and -antinociception in acute pain models. Surprisingly, it enhanced nicotine-induced hypothermia via  $\alpha 7$  nAChRs. In the carrageenan inflammatory test both NS1738 and PNU-120596 significantly reduced thermal hyperalgesia, while only PNU-120596 significantly reduced edema. Importantly, PNU-120596 reversed established thermal hyperalgesia and edema induced by carrageenan. In the chronic neuropathic pain (CCI) model, PNU-120596 had long-lasting (up to 6 hrs), dose-dependent anti-hyperalgesic and anti-allodynic effects after a single injection, while NS1738 was inactive. Subcutaneous and intrathecal administration of MLA reversed PNU-120596's effects, suggesting the involvement of  $\alpha 7$  nAChRs. Finally, PNU-120596 enhanced an ineffective dose of selective agonist PHA-543613 to produce anti-allodynic effects in the CCI model. Our results show a fundamental in vivo difference between type I and II  $\alpha 7$  nAChR PAMs, and demonstrate type II's potential for the treatment of chronic inflammatory pain.

## CHAPTER I

### 1.1 Review of the Literature

Pain, an unpleasant sensation, is one of the most prominent reasons for patient visits to physicians. It is a vital function of the nervous system and an alert mechanism to protect individuals from actual or potential bodily injury. While pain conditions and their treatments vary, the management of chronic pain represents a significant public health issue in the United States. Estimates of its prevalence on population-based surveys vary widely, from 14.6 to 64% (Hardt et al., 2008; Portenoy et al., 2004 and Watkins et al., 2008). By definition, chronic pain is pain that has lasted for a long time (Zhuo, 2007). While the distinction between acute and chronic pain has traditionally been determined by an arbitrary interval of time since onset, most researchers and clinicians used 3 months and 6 months since onset as the most commonly used markers. Characterized by the constant firing of pain signals in the nervous system, chronic pain can last up to years. These prolonged episodes of pain signaling can be due to an initial infliction that has resulted in a long-term lesion or an ongoing infliction that causes acute or chronic lesions. For example, long-term health conditions such as cancer, arthritis, peripheral or central nerve damage and psychological disorders all cause chronic pain conditions. Other common chronic pain conditions include headaches and low back pain. Chronic pain conditions may also occur simultaneously as seen in interstitial cystitis, vulvodynia, fibromyalgia, and endometriosis (“National institute of”, 2012). Chronic pain, which is composed of nociceptive, inflammatory and neuropathic components (Blackburn-Munro, 2004), differs from acute pain in its onset and duration, and by corollary, in its underlying biological mechanisms (Attal & Bouhassira, 1999). Currently employed analgesics are in general less effective in treating chronic pain than in treating acute pain (Wang and Wang, 2003), making the management of chronic pain costly to

the health care system and distressing to the patient. While chronic pain conditions differ, chronic neuropathic pain usually is defined as pain initiated by primary lesion or dysfunction in the nervous system (Harden, 2005). It is characterized by spontaneous pain, such as lancinating, burning, or cramping, among others, and evoked pain, such as allodynia and hyperalgesia (Serra, 1999). Hyperalgesia refers to the exaggeration of pain response produced by a normally painful stimulus, while allodynia is pain produced by normally innocuous stimuli (Calmels et al., 2009; Vranken, 2009).

## **1.2 Pain Modalities**

There are three commonly described etiologies of pain: nociception, inflammation, and neuropathy.

Nociceptive pain is a response triggered by an unpleasant or potentially damaging stimulus activated through nociceptive primary afferents fibers, which generally have higher thresholds than the sensory afferents that signal innocuous touch or temperature information (Walker et al., 1999). This can be acute (such as acute postoperative pain) (Serra et al., 1999 and Woolf et al., 1999) or chronic (such as the inflammation of arthritis).

Inflammatory pain is precipitated by an insult to the integrity of tissues at a cellular level and is mediated by a multitude of reactive cytokines. These chemical signals can directly affect nociceptors or may sensitize them to touch or movement, even some distance from the inflammatory field. In this way one inflammatory mediator may sensitize distant pain receptors for another inflammatory mediator (Feghali and Timothy, 1997). The local inflammatory response is characterized by the active macrophagic release of proinflammatory cytokines,

including IL-10, IFN- $\gamma$  and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), which play a crucial role in triggering the local inflammatory response (Baumann and Gauldie, 1994; Tracey, 2002).

Neuropathic pain is caused by a clinically diverse group of disorders that originate from primary peripheral nerve lesions and/or from a dysfunction in the CNS, often without pain producing stimuli (Harden, 2005).

While acute pain has been effectively managed with opioid and nonsteroidal anti-inflammatory analgesics agents, chronic neuropathic pain associated with inflammation and nerve injury often do not responds well to these or any other drugs (Karlsten & Gordh, 1997; Mannion & Woolf, 2000; Arner & Meyerson, 1988). However, several recent studies have identified the potential use of nicotinic agents for analgesia. Therefore, research that seeks to improve the mechanistic understanding of nicotinic system in the context of chronic pain treatment is both essential and promising for the advancement of clinical chronic pain therapies.

### **1.3 Animal Models of Pain and Inflammation**

Over the past several decades, the mechanisms of nociception and analgesia have been studied clinically and in animal models, namely via biochemical, pharmacological, electrophysiological and behavioral apparatus (Aceto et al., 1986; Mattila et al., 1968). Thus, the use of animal models has been integral in further understanding and identifying other agents and mechanisms with which produce analgesic effects. It has been shown that similar brain structures are involved in the process of nociceptive behaviors in humans and animals (Chang & Shyu 2001), therefore animal models can serve as useful systems with which to study pain. Given the need for novel analgesic agents with superior efficacy, the use of animal models to identify and

validate novel molecular targets and approaches for managing pain, across its different modalities, remain a primary focus.

Progress in pain research has been aided by the development of various animal models that mimic the different modalities of pain. Animal models of nociception should ideally display specificity, sensitivity, validity, reliability, and reproducibility (Le Bars et al., 2001). Of the several models that have been developed to meet these standards, the application of an acute thermal radiation to the tail in the tail-flick test, the application of heat to the paw in the hot plate test, the injection of formalin, the injection of carrageenan and chronic constriction injury are among the most traditionally and well described models. Each of them has its limitations, therefore the interpretation of results from these test must be carefully done. We choose these models based on several criteria. First, these models are amendable for use in mice. This is important because genetically modified nicotinic receptors are only available in mice. Secondly, we used different modalities of pain, such as acute nociceptive, inflammatory and neuropathic pain. Finally, we chose to use these models because they are well described and used traditionally to explore novel drugs.

### 1.3.1 Acute Thermal Pain Tests

#### 1.3.1.1 *Hot-Plate Test*

Usually employing a metallic, heated plate at [52-55°C], the hot plate test measures the latency time until paw-licking, which is one of two consistent supra-spinally-associated behaviors in this test in mice; the other is jumping. One major advantage to the hot-plate test is that its specificity and sensitivity can be altered by choosing which of the two behaviors to measure and by changing the temperature, respectively. Notably, the hot plate test is susceptible

to learning after repeated stimulation and it can be altered by drug that change locomotor activity of the mice (Le Bars et al., 2001).

### 1.3.1.2 *Tail-Flick Test*

Originally developed for use on human subjects (Hardy et al, 1940), the tail-flick test involves the application of thermal radiation to the tail radiant heat or using emerging of the tail of an animal, (Le Bars et al., 2001) thus provoking tail-withdrawal by a quick, strong movement some time after heat application referred to as tail-flick latency. Though the tail-flick is a spinal reflex, it may not always be purely spinal, since it is subject to control by supraspinal structures. Notably, a weakness of the tail-flick test is that it is subject to habituation after repeated stimulation (Le Bars et al., 2001). However this test has been traditionally used with opiates and is widely used in testing new drugs (Martin et al., 1999).

### 1.3.2 Acute Mechanical Pain Test

In animal models of neuropathic pain, mechanical allodynia and sensitivity have most commonly been tested using von Frey filaments, which has been shown to be reliable and valid and humans and in animals (Schmidt et al., 1995). Also called Semmes-Weinstein monofilaments, von Frey consists of 20 carrier-mounted Nylon monofilaments of about 40 mm lengths and diameters of 0.064-1.143 mm. The filaments are rated for their degree of applied force (in terms of ten times the 10-base logarithm of this force). Conceptually, von Frey filaments utilize calibrated and graduated forces to a sensory field (Lambert et al., 2009). There are two applications of the von Frey filaments test, up-down and ascending order (Detloff et al., 2010). Von Frey filaments may largely test cutaneous sensibility and not sensitization produced by deeper tissues (Brennan et al., 1996). We choose the up-down method because studies have

shown that in neuropathic rats the up-down paradigm displayed high reproducibility (Chaplan et al., 1994).

### 1.3.3 Persistent Pain Model

The formalin test for nociception, which is predominantly used with rodents, validly and reliably, induces moderate continuous pain (1 – 2 hr nociceptive behavior) generated by lesioned tissue (Dubuisson & Dennis 1977; Franklin & Abbott, 1989; Tjolsen et al., 1992). In this way it differs from acute tests of nociception, which rely upon brief stimuli of threshold intensity. The response to formalin is biphasic, consisting of early and late phases. The early phase is caused predominantly by C-fiber activation due to the peripheral stimulus, while late phase appears to be dependent on the combination of an inflammatory reaction in the peripheral tissue and function changes in the dorsal horn of the spinal cord.

### 1.3.4 Inflammatory Pain Model

There are three commonly used models of intraplantar injection-induced inflammatory pain – lipopolysaccharides (LPS), Complete Freund's Adjuvant (CFA) and carrageenan. LPS is a gram negative bacteria cell wall constituent that has been found to induce long-lasting hyperalgesia (Qanaan et al., 1996). CFA is composed of inactivated and dried mycobacteria (Ren & Dubner, 1996). The CFA injection produces an intense inflammation after each behavior and hyperalgesia develops 3 to 4 h after injection, peaks at 6 to 24 h and lasts for more than 5 days (Ren & Dubner, 1993). Carrageenan is a polysaccharide that is extracted from red seaweed. There are three main commercial classes of carrageenan – kappa, iota and lambda, which differ in their constituent chemical group positions (Thomson & Fowler, 1981). The carrageenan model can be used as a method to assess short-duration (1 – 3 days) inflammatory pain. The



inflammatory response has been shown to last up to 6 to 8 h after injection and increases in its duration with the dose (Morisseau et al., 2010; Hroach & Sufka, 2003). While various model of inflammation are described we decided on the carrageenan test. An intraplantar injection of carrageenan stimulates local inflammation, inducing edema of the paw tissues (Huang et al., 2012). It also induces inflammation associated with mechanical allodynia and c-fos-protein-like immunoreactivity in the ipsilateral dorsal horn of the spinal cord (Winter et al., 1962; Kocher et al., 1987; Kayser et al., 1987). This model provides rapid assessment of a compound's ability to provide anti-inflammatory and analgesic activity. Agents that show activity in this assay include NSAIDs, glucocorticoids and cox inhibitors.

#### 1.3.5 Neuropathic Pain Model

There are three commonly used models of chronic neuropathic pain – the partial sciatic nerve ligation model (PSL), spinal nerve ligation model (SNL), and the chronic constriction injury (CCI). For all three, allodynia and hyperalgesia develops quickly after ligation and lasts for more than two months, though SNL requires the most extensive surgical procedure of the three. CCI is reported to be the most seemingly frequently used model, and it has been validated by being shown to induce both thermal hyperalgesia and mechanical allodynia for at least seven weeks post-surgery (Vry et al., 2004). It is important to note that in CCI, an inflammatory action develops after a loss of most A-fibers and some C-fibers. This inflammatory component is interesting for us because of the  $\alpha 7$  nAChR is associated with the cholinergic anti-inflammatory pathway in the termination of the parasympathetic nervous system (Xiong et al., 2010). Moreover, in the CCI model, we measured allodynia and thermal hyperalgesia (Bridges et al., 2001).

#### 1.4 Available treatments and challenges

Despite a growing understanding of and an increasingly effective treatment for chronic pain conditions, these conditions are still poorly managed clinically. At present, opiate analgesics remain the standard of treatment for the majority of chronic pain conditions, however issues relating to tolerance, physical and psychological dependence and side effects such as respiratory depression, constipation and nausea, are characteristic of this drug class. Pain relief for patients with inflammatory disorders through the use of non-steroidal anti-inflammatory drugs (NSAIDs), such as celecoxib, has had moderate success (Tramer et al., 1998; Flower, 2003). While gastrointestinal adverse effects have traditionally been considered the most common and worrisome complications, the risk of cardiovascular complications have increasingly gained attention in the last decade. Finally, drugs that stabilize or moderate CNS function, such as drugs prescribed for seizures and depression may treat chronic neuropathic pain more effectively (Karlsten & Gordh, 1997; Mannion & Woolf, 2000). In recent times, chronic neuropathic pain, which often includes inflammatory and nociceptive aspects (Attal, 1999), is symptomatically treated with anticonvulsants and antidepressants such as gabapentin and amitriptyline (Lee et al., 2011). The drawback of these drugs is their adverse side effect profile (sedation, abnormalities of blood or platelet counts; anti-cholinergic side effects).

While the current treatments offer some relief, adverse effects and low efficacy are still the major drawbacks for the treatment of chronic neuropathic pain conditions. Therefore, the improvement of chronic neuropathic pain will require a better understanding of the mechanisms underlying this condition and of the cause-and-effect factors to test the pharmacological agents that address this issue.

## 1.5 Nicotinic Acetylcholine Receptors

Nicotinic acetylcholine receptors (nAChRs) are known to play a significant role in pain transmission. This was first evidenced by reports from animal studies that found nicotine and epibatidine (nonselective nicotinic agonists) to be effective and potent antinociceptive drugs in acute and chronic pain models (Damaj et al., 1998 and Tripathi et al., 1982).

However, the use of these nonselective nicotinic receptor agonists is limited by side effects and challenges, including hypothermia, motor impairments and lack of therapeutic window (Bannon et al., 1995; Kesingland et al., 2000 and Rowbotham et al., 2009). For example, the antinociceptive dose of epibatidine nearly caused seizure in rodents (Sullivan et al., 1994). The significant toxic effects of epibatidine were due to its non-selective actions on a broad range of nAChR subtypes, in particular those localized to peripheral ganglionic junctions. Therefore, in order to develop safe and effective analgesic nicotinic agonists, selective ligands for nAChR subtypes that are implicated in modulating nociceptive transmission are required.

Pain transmission studies have largely implicated two nicotinic receptor subtypes in the past decade:  $\alpha 4\beta 2^*$  and  $\alpha 7$ . Due to its abundance in the central nervous system (CNS), the most commonly researched nAChR subtypes is the  $\alpha 4\beta 2^*$  receptor. Appreciation of the involvement of this receptor in pain has come from selective agonist and antagonist studies as well as the use of genetic knockouts in animals and antisense RNA (Bitner et al., 1998; Decker et al., 2004; Marubio et al., 1999; Jackson et al., 2010). However, another major subtype also with evidence suggesting a role in mediating nicotinic antinociception is the  $\alpha 7$ -homomer nicotinic subtype.

### 1.5.1 Subunit Composition, Stoichiometry and Distribution

The specific pharmacological response, such as affinity and efficacy of nicotinic agonists, calcium permeability and rate of receptor desensitization (Gotti and Clementi, 2004; Gerzanich et al., 1998; Boorman et al., 2003) evoked by a nAChR agonist is governed by the anatomical distribution and expression of each receptor subtype and by the stoichiometry of subunits comprising each subtype.

nAChRs are pentameric structures composed of five different subunits that form a central ion-conducting pore, allowing the permeability of cations, such as sodium, potassium, and calcium. As nicotine binds to the ligand-gated ion channels it causes different conformational states in the nAChR: activation (open) and subsequent desensitization (closed and incapable of re-activation), then a basal resting state (closed and capable of activation). nAChRs are present in both the peripheral nervous system (PNS) (at the skeletal neuromuscular junction and in the autonomic nervous system) and the CNS. The neuromuscular nAChR is composed of  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ , and  $\epsilon$  subunits, whereas the neuronal nAChR is composed of subunits  $\alpha 2$ - $\alpha 10$  and  $\beta 2$ - $\beta 4$ . Indeed, neuronal nAChRs expressed in the CNS mediate fast synaptic transmission and regulate processes such as neurotransmitter release, synaptic plasticity and neuronal network integration by providing modulatory input to a variety of other neurotransmitter systems.

nAChRs have distinct homomeric or heteromeric subunit compositions. Consequently, each subunit composition may have different functional properties that result in various physiological and pharmacological effects (Millar and Gotti., 2009). Homomeric nAChRs are made up of  $\alpha 7$ ,  $\alpha 8$  or  $\alpha 9$  subunits, while heteromeric nAChRs comprise various combinations of  $\alpha 2$ - $\alpha 6$  with  $\beta 2$ - $\beta 4$  or  $\alpha 9$  with  $\alpha 10$  subunits (McGehee., 1999 and Dani et al., 2001). The  $\alpha 5$  and

$\beta 3$  subunits are believed to be modulatory since they do not form functional receptors when expressed alone or when expressed as a single  $\alpha$  or  $\beta$  subunit in heteromeric channels. On the other hand, both of these subunits alter agonist affinity, calcium permeability and desensitization when expressed with other functional channels (Gerzanich et al., 1998; Boorman et al., 2003). Typically, activation of brain nAChRs results in an enhanced release of various neurotransmitters, including dopamine, serotonin, glutamate and  $\gamma$ -aminobutyric acid (GABA).

The two most abundant nAChRs in the CNS can be differentiated by their relative affinities for nicotine and  $\alpha$ -bungarotoxin. nAChRs with high affinity for nicotine but low affinity for  $\alpha$ -bungarotoxin largely contain combinations of  $\alpha 4$  and  $\beta 2$  subunits, while nAChRs with low affinity for nicotine but high affinity for  $\alpha$ -bungarotoxin are mainly  $\alpha 7$ -containing.  $\alpha 3\beta 4$  subunits are less abundant in the CNS being widespread in the autonomic nervous system as well as responsible for several of the nicotine adverse effects (Lee et al., 2011). One of the major problems to the progress of specifically targeted nicotinic agonists is a need of knowledge concerning the exact subunit composition of native nicotinic receptors. It has been revealed that nAChRs are implicated in a variety of physiological functions such as modulating neurotransmission, cognition, development, anxiety and nociception (Cordero-Erausquin et al., 2000; Lloyd & Williams, 2000).

### 1.5.2 Nicotinic Receptors in Nociceptive Systems

The presence of nAChRs appears to not be homogeneous across pain conduction pathways (Khan et al., 2003). Nicotinic receptors are found on primary afferents (Genzen & McGehee, 2003; Li et al., 1998) and spinal dorsal horn (Cordero-Eraudquin et al., 2004). Previous studies suggest that primary afferent C-fibers express  $\alpha 4\beta 2^*$  and  $\alpha 7^*$  nAChRs (where asterisks indicate additional subunits). These receptors are probably responsible for nociceptive

responses while  $\alpha 3\beta 4^*$  may be responsible for antinociceptive properties (Khan et al., 2001; Rueter et al., 2000). Notably, nociceptive and antinociceptive responses can be attributed to different nAChR subtypes (Rueter et al., 2000). Furthermore, nAChRs are broadly expressed in the rat spinal cord, modulating innocuous and nociceptive transmission (Young et al., 2008).

The spinal dorsal horn is the initial location in the CNS where somatosensory input is received, integrated and transmitted. Multiple subtypes of nAChRs are expressed in the spinal dorsal horn, with a predominance of  $\alpha 4\beta 2^*$  and  $\alpha 7$  subtypes (Cordero-Eraudquin et al., 2004; Changeux et al., 2004). The receptor subtypes that mediate central antinociceptive action of nicotine are largely unknown. The  $\alpha 4\beta 2$  and  $\alpha 7$  subunits are strong candidates for central antinociceptive mediation because of their distribution and abundance in neuronal pathways (Gillberg and Aquilonius, 1985; Khan et al., 1994; Marks et al., 1992; Seguela et al., 1993; Wada et al., 1989). In the dorsal horn the majority of inhibitory GABAergic and/or glycinergic interneurons preferentially express  $\alpha 4$ ,  $\alpha 6$  and  $\beta 2$  subunits whereas excitatory neurons mainly express  $\alpha 7$ ,  $\alpha 3$  and  $\beta 2$  subunits (Cordero-Eraudquin et al., 2004). However, Cordero-Eraudquin & Changeux (2001) have shown that endogenous acetylcholine (ACh) may tonically activate the nAChRs present on inhibitory interneurons in the dorsal horn.

The spinal cholinergic system has been indicated to modulate sensory inputs from the periphery (Khan et al., 1994) and are found to be expressed on both inhibitory and excitatory interneurons in the spinal dorsal horn (Cordero-Eraudquin et al., 2004). The activation of presynaptic nAChRs facilitates GABAergic and glycinergic inhibitory synaptic transmission in the superficial dorsal horn (Kiyosawa et al., 2001; Genzen & McGehhee, 2005). nAChRs have also been shown to exert tonic control on descending inhibitory noradrenergic (Li et al., 2000) and serotonergic transmission (Cordero-Eraudquin & Changeux., 2001). Previous studies have

demonstrated that the administration of nicotinic agonists results in enhanced neurotransmission in the dorsal horn of the spinal cord (Genzen & McGehee, 2005; Takeda et al., 2003; Rashid et al., 2006). Furthermore, other studies have shown that partial sciatic nerve injury in the mouse results in an increased spinal antinociceptive potency of nicotinic agonists and loss of cholinergic stimulated GABAergic inhibitory tone at  $\alpha 4\beta 2$  nAChRs (Rashid & Ueda, 2002; Rashid et al., 2006). Even though the effect of exogenous cholinomimetic drugs, such as nicotine and epibatidine, on nociceptive transmission in the spinal cord has been studied (Cordero-Erausquin & Changeux 2001, 2004; Rashid et al., 2006), the role of endogenous ACh in nociceptive processing continues to be vague. Some have proposed that ACh contributes to the analgesic action of opioids and of the  $\alpha 2$ -adrenoceptor agonist clonidine, which also has intrinsic analgesic properties (Chen & Pan, 2001; Hood et al., 1997; Pan & Chen, 1999). In a previous study Rashid & Ueda (2002) demonstrated that intrathecal (i.t.) injections of mecamylamine, a nicotinic antagonist produces thermal hyperalgesia in sham-operated mice, suggesting the presence of a tonic inhibitory mechanism through nicotinic receptors in the spinal cord. The exploitation of  $\alpha 7$  nAChRs' role in inhibiting nociceptive pathways may be an important way by which to manage pain in animal models.

### 1.5.3 $\alpha 7$ Nicotinic Receptors in Pain Pathways

The nAChR  $\alpha 7$  homopentameric structure is characterized by its high calcium permeability, the blockade by selective antagonists such as  $\alpha$ -bungarotoxin and methyllycaconitine (MLA), and the rapid desensitization after agonist stimulation (Feuerbach et al., 2008). The  $\alpha 7$  nAChR has a high permeability to calcium, especially when compared to other nAChRs and NMDA receptors (Seguela et al., 1993; Castro & Albuquerque, 1995). Williams et al. (2011) points out that there are several essential characteristics for the  $\alpha 7$  subtype that

differentiate it from the heteromeric nAChRs, specifically citing higher energy barriers for entering the open state and the low open state stability, a unique desensitized state, and a relatively small energy difference between the resting and desensitized states. It has been demonstrated that the nAChR can change conformation from the desensitized to the resting state without passing through the active state. For example, upon desensitization,  $\alpha 7$  nAChRs do not convert to a state with high affinity for ACh. Once ACh has been removed or metabolized, the receptor rapidly desensitizes and can be activated again by ACh (Papke et al., 2009). However  $\alpha 7$  nAChRs do not readily return to a functional state after the application of nicotine or the  $\alpha 7$  agonist anabaseine (Papke et al., 2000). Furthermore, brief applications of endogenous  $\alpha 7$  agonist choline, at high concentrations, can transiently activate  $\alpha 7$  subtypes, lower steady-state concentrations induce pre-desensitization and down-regulate response to ACh (Papke et al., 2009).

In the CNS,  $\alpha 7$  nAChRs are expressed in the cortex, hippocampus, lateral geniculate nucleus, superior colliculus, striatum and the dorsal horn of the spinal cord. Supporting this, Stephen et al. (2008) showed for the first time that a selective  $\alpha 7$ -nAChR agonist had the ability to raise CFA-induced paw withdrawal thresholds and increase weight bearing in both rat and mouse through a primarily central mechanism. At the cellular level we find  $\alpha 7$  nAChR are both present pre- and post- synaptically on GABA glutamate and cholinergic-neurons as well as on astrocytes and neuroglia (Feuerbach et al., 2008). Interestingly, animal models of inflammatory and chronic neuropathic pain demonstrate a host of changes in protein expression and function in the nociceptive pathway, such as the upregulation of the  $\alpha 7$  subunit in the dorsal root ganglia (DRG) (Xiao et al., 2002).



In vitro, nicotine is neuroprotective against glutamate-induced toxicity via  $\alpha 7$  nAChRs (Gahring et al., 2003). This effect is mediated by the activation of both  $\alpha 4\beta 2$  and  $\alpha 7$  nAChRs (Kaneko et al., 1889; Hejmadi et al., 2003). Previous studies have shown that the release of glutamate in the spinal cord following peripheral nerve injury and the subsequent loss of spinal cord neurons are believed to be key mediators in the development of chronic pain (Whiteside & Munglani, 2001). Therefore, the  $\alpha 7$  nAChR subtype appears to be a potential target for pain mediation.

#### 1.5.4 $\alpha 7$ Nicotinic Receptors in the Cholinergic Anti-Inflammatory System

Recent evidence has suggested that the cholinergic anti-inflammatory pathway regulates the immune system. This neurophysiological mechanism reduces inflammation by decreasing cytokine synthesis via release of ACh in organs of the reticulo-endothelial system, such as the lungs, spleen, liver, kidneys and gastrointestinal tract (Bencherif et al., 2011). Cholinergic action can control the immune system via the vagus nerve; this central mechanism is dependent on the  $\alpha 7$  nAChR subtype (Tracey Kj, 2007; Rosas-Ballina & Tracey Kj, 2009; Wang et al., 2003). Both muscarinic and nicotinic cholinergic receptors such as  $\alpha 7$  nAChRs, are expressed on T cells, macrophages and other immune cells which are capable of producing ACh. B and T cells are regulated by ACh via different cholinergic receptors (Fujii et al., 2008; Kawashima and Fujii, 2003). Previous studies have demonstrated the importance of ACh in down regulating proinflammatory cytokine synthesis and preventing tissue damage by directly interacting with  $\alpha 7$  nAChRs expressed on macrophages and other cytokine-producing cells (Tracey, 2002; Wang et al., 2009).  $\alpha 7$  nAChRs are present on immune and non-immune cytokine-producing cells, including macrophages and keratinocytes (Wang et al., 2003; Pavlov and Tracey, 2004). The modulation of inflammation by macrophagic  $\alpha 7$  nAChRs (Wang et al., 2003) is mediated by

ACh through the vagus nerve, which acts on  $\alpha 7$  nAChRs on macrophages to suppress the release of TNF- $\alpha$  and other proinflammatory cytokines (De Simone et al., 2005; van Weterloo, 2010). Indeed, this anti-inflammatory effect reflects the actions of endogenously released ACh on  $\alpha 7$  subtypes.

Watkins et al (2001) have identified the role of pro-inflammatory cytokines in the development and maintenance of chronic pain conditions. Macrophages present in the periphery release the pro-inflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$ , which contribute to behavioral hypersensitivity following peripheral nerve injury (Bendszus & Stoll, 2003; Mueller et al., 2003). Shytle et al (2004) demonstrated that nicotine pretreatment of cultured microglial cells inhibited LPS-induced TNF- $\alpha$  release via  $\alpha 7$  nAChRs. The maintenance of chronic pain is regulated by macrophages, spinal microglia and astrocytes, which produce pro-inflammatory cytokines. Additional studies with  $\alpha 7$  subunit knockout mice reveal a critical role for the  $\alpha 7$  nAChRs as a peripheral component of this cholinergic anti-inflammatory system (Wang et al., 2003). The efficacy of  $\alpha 7$  nicotinic agonists in the setting of inflammation could be a result of modulation of the release of cytokines by local macrophages. For example, choline reduces TNF release from macrophages by activating the  $\alpha 7$  nAChR (Wang et al., 2003). Furthermore, in chronic inflammation there is evidence of the efficacy of cholinergic modulation of microglial activation by  $\alpha 7$  nAChRs (Shytle et al., 2004). Thus, both directly, through the pain pathway, and indirectly, through the cholinergic anti-inflammatory pathway, the  $\alpha 7$  nAChR presents itself as a feasible target for pain modulation.

## 1.6 Positive and Negative Allosteric Modulation of nAChRs

Modulation of nAChR function has been achieved by a variety of pharmacological mediators termed allosteric modulators. An alternative approach to enhance  $\alpha 7$  nAChR function is by augmenting effects of the neurotransmitter ACh via positive allosteric modulation that can reinforce the endogenous cholinergic neurotransmission without directly activating  $\alpha 7$  nAChRs. These bind to protein regions other than the active, or orthostatic, site, such as the allosteric site. Allosteric sites are distinct from orthosteric sites and can be localized elsewhere on the protein. Binding of an agonist ligand at the orthosteric site stabilizes the protein in the active state, while binding of a modulator molecule at an allosteric site allosterically modulates the shape of the protein. The protein transition state might be affected by the binding of an allosteric ligand resulting in a displacement of the equilibrium between states. Modulator molecules that are able to lower the energy barrier between the resting and active states enhancing the agonist-evoked response are referred as positive allosteric modulators (PAMs). In contrast, modulator molecules that increase this energy barrier will cause a decrease of the agonist response and are referred to as negative allosteric modulators (NAMs) (Bertrand & Gapalakrishnan, 2007). Positive and negative allosteric modulators are able to activate or inhibit a pharmacological effect through a receptor-mediated response (Faghih et al., 2007; Davey et al., 2012).

PAMs are compounds that facilitate endogenous neurotransmission and/or enhance the efficacy and potency of agonists without directly stimulating the agonist binding sites. In addition, a PAM alone, in principle, does not exhibit intrinsic activity at the receptor, however it can amplify the effects of an agonist; these characteristics were first identified in ligand-gated ion channel receptors, including nAChRs (Alkondon et al., 1997; Faghih et al., 2007). A small

number of such compounds have been characterized *in vivo*. Benzodiazepine, a psychoactive compound, is an example of a PAM approach to enhance GABA<sub>a</sub> receptor channel activity and has been shown to be clinically successful (Hevers & Luddens, 1998).

ACh is a neurotransmitter in both the PNS and the CNS. In the PNS, ACh activates muscles, and is a major neurotransmitter in the autonomic nervous system. In the CNS, ACh structures the cholinergic system, which tends to cause inhibitory actions. ACh is produced by the enzyme choline acetyltransferase from the compounds choline and acetyl-CoA. The enzyme acetylcholinesterase converts ACh into the metabolites choline and acetate. Choline is a naturally occurring endogenous nucleoside used in the synthesis of the constructional components in the body's cell membranes, an essential nutrient for the normal function of the cells (Aslan et al., 2011), and precursor for the synthesis of the cholinergic neurotransmitter ACh. Furthermore, choline is a selective natural ligand for  $\alpha 7$  nAChR and its therapeutic significance is limited because of its low potency ( $EC_{50} \sim 0.5-1.6\text{mM}$ ) in neurons cultured from the rat hippocampus, olfactory bulb and thalamus as well as in PC12 cells (Alkondon et al., 1997; Papke & Porter Papke, 2002). Exogenous choline has been known to exhibit antinociceptive effects in various animals models of pain when administrated i.t., intravenously (i.v.), intracerebroventricularly (i.c.v.) or systemically (Damaj et al., 2000; Wang et al., 2005; Rowley et al., 2010). Earlier studies, in mice, show that choline, coadministered with aspirin, significantly decreases late-phase licking/biting times in the formalin test at doses that produce no effects on this response when either drug is administered alone (Wang et al., 2005). Choline also significantly increases morphine antinociception during the late phase of the formalin test (Wang et al., 2005). The effect of choline on anti-inflammatory pain was blocked by the selective  $\alpha 7$  antagonist MLA but

not by macamylamine, naloxone or atropine, suggesting that choline's anti-inflammatory effects were  $\alpha 7$  receptor-mediated (Wang et al., 2005).

### 1.6.1 Advantages of Using Positive Allosteric Modulators

A potential advantage in using PAMs, rather than direct agonists, is selectivity, since PAM binding sites are likely different from agonist/competitive antagonist binding sites. As a result, the determination of PAM selectivity is important to avoid potential non- $\alpha 7$  nAChR interactions (Gronlien et al., 2007). Allosteric modulators usually have low intrinsic activity although they provide selective potentiation or inhibition of physiological activity, lacking direct interruption of the ongoing signaling processes (Taly et al., 2009). One potential effect would be to enhance agonist-binding to the resting state of the receptor, increasing potency. This was proposed to be the mechanism for benzodiazepine enhancement of GABA<sub>a</sub> receptor function (Doble 1999).

The  $\alpha 7$  nAChR is both activated and desensitized by agonists in a concentration-dependent manner, and they can become desensitized at agonist concentrations lower than those required to substantially activate the receptor (Quick & Lester, 2002). Thus, under conditions of chronic treatment,  $\alpha 7$  nAChR agonists may have a relatively narrow effective concentration, supporting the use of PAMs, which can be effective at a wide range of concentrations (Christopoulos, 2002). Several explanations may account for this narrow effective concentration of  $\alpha 7$  nAChR agonists. First, long-term drug exposure could lead to tolerance to analgesic effects and to prolonged receptor desensitization after agonist-binding (Papke et al., 2000; Christopoulos, 2002). For example, Gao et al (2010) found no analgesic efficacy with agonists selective for  $\alpha 7$  in formalin-evoked paw flinching. Second, Christensen et al (2010) points out

that chronic exposure to  $\alpha 7$  agonists induced an upregulation of  $\alpha 7$  nAChRs in the brain leading to coupling and uncoupling in specific intracellular signaling pathways. Third, the development of  $\alpha 7$  nAChR selective agonists is further hindered by a high structural homology between  $\alpha 7$  and 5-hydroxytryptamine (5HT<sub>3</sub>) receptors (Eisele et al., 1993; Gao et al., 2010). Finally, most of the  $\alpha 7$  agonists, such as SSR18711, AZD-0328, S24795, SEN-12333, and JN-403, show partial agonist activity (i.e., maximum efficacy <75% versus ACh) and/or relatively weak potency (Ding et al., 2012; Sydserff et al., 2009).

Another significant benefit of PAMs is that only receptors activated by the endogenous ligand are subject to modulation. Consequently, the physiological characteristics of endogenous receptor activation are conserved, and the function of the modulator can be measured as increasing the gain of individual receptor activation events. Agonists, on the contrary, tonically activate all receptors, leading to non-physiological patterns of receptor activation that could be detrimental to corresponding systems. Thus, using PAMs as an alternate agent through which to activate nAChRs could serve as a way to elicit unique, favorable enhancements of physiological responses, such as pain attenuation.

### **1.7 Allosteric Modulation of $\alpha 7$ nAChRs**

A number of chemically diverse PAMs have been recognized and largely classified at least into two groups based on several distinctive qualities, such as differential effects on ionic current responses and reactivation of desensitized  $\alpha 7$  nAChRs (Gronlien et al., 2007). PAMs have been classified as either type I, such as NS1738, or type II, such as PNU-120596, on the basis of their different effects on desensitization (Bertrand & Gopalakrishnan, 2007; Timmermann et al., 2007; Collins et al., 2011). The primary difference between these two types

is in their ability to evoke a response at the receptor level. The PAMs classified as type I predominantly affects the apparent peak current with little effect on desensitization kinetics whereas type II increases the apparent peak current and evoke a distinct weakly decaying current (Hurst et al., 2005). Timmermann et al (2007) reported that NS1738 enhances the potency of ACh as well as its maximal efficacy. Interestingly, a separate study shows that NS1738 and PNU-120596 are aromatically-linked urea compounds and that both of them interact competitively at a common transmembrane (TM) site (Collins et al., 2011). Usually, the  $\alpha 7$  transmembrane region is the site of action for a variety of allosteric modulators, however there is strong evidence that allosteric modulators can interact with sites on the nAChR extracellular N-terminal domain such as galantamine (Collins et al., 2011), and selective  $\alpha 7$  PAMs.

The PAM PNU-120596 binding site location on  $\alpha 7$  nAChRs might be in the transmembrane domain (TMD) (Young et al., 2008; Bertrand & Gopalakrishnan, 2007). It comprises five amino acids within TM1, TM2 and TM4 domains which, when mutated, significantly diminish  $\alpha 7$  nAChRs allosteric modulators potentiation. These amino acids structure an intrasubunit cavity that is situated between the four TMDs and might be alike to the binding site for neurosteroids and volatile anesthetics on members of the ligand-gated ion channel family, including GABA<sub>A</sub> and glycine receptors (Witschi et al., 2011). In addition to this property, PNU-120596 has been demonstrated to act as a PAM of nAChRs both in vitro and in vivo by electrophysiology studies (Hurst et al., 2005).

The in vitro effects of PNU-120596 were shown to be mediated by  $\alpha 7$  nAChRs. This compound increases the apparent peak  $\alpha 7$  current response and strongly affects the time course of current response. At the single-channel level, PNU-120596 increases mean open time but does not alter the pattern of the physiological response (Hurst et al., 2005). On the other hand,

systemic administration of PNU-120596 to rats improved the auditory-gating deficit caused by amphetamine, showing, for the first time, an *in vivo* efficacy for an  $\alpha 7$  nAChR PAM (Hurst et al., 2005).

PNU-120596 facilitates and/or enhances nicotinic cholinergic neurotransmission in large part due to destabilizing desensitized states; on their own, they cannot desensitize nAChRs (Buccafusco et al., 2008). A recent study demonstrated that PNU-120596 enhances the effects of subthreshold concentrations of choline on native  $\alpha 7$  nAChR using patch-clamp electrophysiology and histaminergic tuberomammillary neurons in hypothalamic slices, allowing physiological levels of choline to activate these receptors and produce whole-cell responses in the absence of exogenous nicotinic agents (Gusev and Uteshev, 2009). In the cerebrospinal fluid (CSF), choline is present with a low concentration (5-10 $\mu$ M) (Sarter and Parikh, 2005; Parikh and Sarter, 2006). It is improbable that in the absence of cholinergic synaptic inputs or exogenous nicotinic agents,  $\alpha 7$  nAChRs are persistently activated or desensitized by endogenous choline (Uteshev et al., 2003). However, the effects may be notably different in the presence of PNU-120596, which significantly enhances the responsiveness of  $\alpha 7$  nAChRs to nicotinic agents (Hurst et al., 2005; Gronlien et al., 2007; Roncarati et al., 2008; Young et al., 2008; Lopez-Hernandez et al., 2009).

The dose-response relationships of PNU-120596 indicate that the EC<sub>50</sub> value for potentiating effects of ACh is near 1.5 $\mu$ M (Gronlien et al., 2007; Young et al., 2008). Furthermore, Gronlien et al. (2007) found that concentration-response to agonist ACh and the two  $\alpha 7$  nAChR agonists GTS-21 and PNU-282987 were potentiated by type I and II  $\alpha 7$  PAMs, although type II PAMs had greater effects in *X. laevis oocytes*. This concentration of PNU-



120596 is readily achievable in the CSF *in vivo*. The concentration of PNU-120596 identified in the rat brains receiving 1mg/kg i.v. was found to be near 1.5  $\mu$ M (Hurst et al., 2005).

Furthermore, PNU-120596 can alter the equilibrium between active and desensitized states resulting in significantly prolonged responses, even promoting the activation of previously desensitized receptors (Gronlien et al., 2007). For example, while continuous exposure to agonist (nicotine) desensitizes the  $\alpha$ 7 nAChR resulting in a decreased response at the receptor level to a non-detectable level, PNU-120596 with chronic administration to nicotine is able to restore a current even larger than the peak current evoked by the agonist alone (Hurst et al., 2005). This attribute does not appear to be specific to PNU-120596, but may be a common feature of type II PAMs (Gronlien et al., 2007). This biophysical property could have implications after chronic exposure of type II PAMs *in vivo*.

The effective tone produced by PNU-120596 appears to be mediated through modulation of the  $\alpha$ 7 desensitized state. Also, this compound has no effect on ion selectivity and produces an insignificant effect on channel conductance (Hurst et al., 2005), sustaining the hypothesis that PNU-120596 stabilizes intrinsic states of the channel. A recent *in vivo* study using rats by Christensen et al (2010) showed that [125]- $\alpha$ -bungarotoxin (BTX) autoradiography on acute or repeated administration of a selective  $\alpha$ 7 nAChR agonist was able to increase the number of  $\alpha$ 7 nAChR binding sites in many brain regions, predominantly in the prefrontal cortex. However, PNU-120596 and NS1738 did not increase [125]-BTX binding, providing an essential difference between repeated administration with agonists and positive allosteric modulators of the  $\alpha$ 7 nAChR.

## 1.8 Thesis Objectives

Research in the last decade suggests that  $\alpha 7$  nAChRs show promise as potential targets for cognitive deficits of the CNS and inflammatory diseases. These studies have mainly explored the role of  $\alpha 7$  nAChRs in animal models of cognitive dysfunction with selective agonists and PAMs. While PAMs have been shown to produce pro-cognitive effects, it is not known whether these agents will provide a significant benefit in pain and inflammation. Therefore, this study aims to investigate whether the enhancement of endogenous  $\alpha 7$  nAChR function will result in a beneficial effect in nociceptive, inflammatory and chronic neuropathic pain models. For that we examined the effects of two classes of  $\alpha 7$  PAMs such as type I (NS1738) and II (PNU-120596) in acute thermal (tail-flick and hot-plate) tests, a tonic (formalin) test, an inflammatory (carrageenan) test and CCI model in the mouse after systemic and central administration of these drugs. NS1738 and PNU-120596 were chosen because of their ability to improve cognitive impairments in rats after systemic administration, demonstrating their efficacious activity

Hurst, et al., 2005; Timmermann et al., 2007). Also, these PAMs have displayed the clinically relevant ability to activate the  $\alpha 7$  nAChR without altering physiological ion gradients, inducing cellular dysfunction due to ionic imbalances, or causing adverse side-effects.

We hypothesize that reinforcing endogenous cholinergic tone through the  $\alpha 7$  nAChR neurotransmission with  $\alpha 7$  PAMs NS1738 (type I) and PNU-120596 (type II) will produce antinociceptive, anti-hyperalgesic and anti-allodynic effects in models of pain in mice. Our first aim was to characterize the pharmacology of these type I and II PAMs by determining the time course and dose-effect of these drugs after systemic and central administration in different pain models. We then identified the nAChR subtypes that mediate the effect of  $\alpha 7$  PAMs and their site of action. Our second aim was to investigate the interactions between PNU-120596 with

both exogenous  $\alpha 7$  nAChR selective and non-selective nicotinic agonists in a model of persistent pain. Third, we examined the enhancement of nonselective agonist nicotine by PNU-120596 in models of acute thermal pain, persistent pain as well as in its effect on body temperature, locomotor activity and seizure activity.

## CHAPTER II

### **The Antinociceptive Effects of Nicotinic Receptors Alpha 7 Positive Allosteric Modulators in Murine Acute and Tonic Pain Models**

#### **2.1 Chapter II Introduction**

In recent years, the  $\alpha 7$  nAChR agonists have received significant attention as possible targets for drug discovery due in part to evidence that they may have a cognitive enhancing role, antinociceptive and anti-inflammatory properties (Damaj et al., 2000; Wang et al., 2005; de Jonge and Ulloa, 2007; Thomsen et al., 2010; Rowley et al., 2010)  $\alpha 7$  nAChR agonists such as choline, CDP-choline, compound B, JN403 and AR-R17779 were found to exhibit anti-inflammatory effects in various inflammation and pain models in rodents (Damaj et al., 2000; Feuerbach et al., 2009; Medhurst et al., 2008; van Maanen et al., 2009; Gurun et al., 2009; Rowley et al., 2010; Marrero et al., 2011). Although  $\alpha 7$  nAChRs agonists have shown beneficial effects in inflammatory animal models in some studies, this effect was not consistently seen in others (Gao et al., 2010).

Recently, several structurally diverse and selective  $\alpha 7$  nAChRs PAMs, including PNU-120596 (Hurst et al., 2005), TQS (Grønlien et al., 2007) and NS1738 (Timmermann et al., 2007) were reported. Both I and II type PAMs have been shown to exhibit cognitive enhancement *in vivo* in rodents. For example, PNU-120596 reversed amphetamine-induced gating deficits in rats and NS1738 improved performance in the rat social recognition (Hurst et al., 2005; Timmermann et al., 2007). While these observations show that  $\alpha 7$  PAMs, belonging to both types, are effective in certain rodent cognitive models, none of these agents has been studied in animal models of pain and inflammation.

Therefore, the present study was designed to investigate the effects of type I and II positive allosteric modulators in acute and tonic pain models in the mouse. PNU-120596 and NS1738 effects were tested after different routes of administration in acute thermal (tail-flick and hot-plate tests) and tonic (formalin test) pain models in mice. Site of actions and receptors mechanisms were also determined. Since  $\alpha 7$  PAMs were reported to enhance extracellular signal-regulated kinase (ERK) signalling in PC12 cells (Hu et al., 2009; El Kouhen et al., 2009), we hypothesized that activation of ERK by these allosteric modulators might play an important role in their antinociceptive effects. Hence, we explored this possibility by examining the effects of U0126, a specific MEK inhibitor (Duncia et al., 1998), on the antinociceptive actions of  $\alpha 7$  PAMs.

## **2.2 Materials and Methods**

### **2.2.1 Animals**

Male ICR mice obtained from Harlan Laboratories (Indianapolis, IN) and male C57BL/6 mice from Jackson Laboratories (Bar Harbor, ME) were used throughout the study. Mice null for the  $\alpha 7$  (Jackson Laboratories) subunits and their wild-type littermates were bred in an animal care facility at Virginia Commonwealth University. For all experiments, mice were backcrossed at least 8 to 10 generations. Mutant and wild types were obtained from crossing heterozygote mice. This breeding scheme controlled for any irregularities that might occur with crossing solely mutant animals. Mice had free access to food and water and were housed in groups of six in a 21°C humidity-controlled animal facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The rooms were on a 12-h light/dark cycle (lights on at 7:00 a.m.). Mice were 8–10 weeks of age and weighed approximately 20–25 g at the start of all the experiments. All experiments were performed during the light cycle (between 7:00 a.m. and 7:00 p.m.) and the study was approved by the

Institutional Animal Care and Use Committee of Virginia Commonwealth University. All studies were carried out in accordance with the National Institute of Health guide for the Care and Use of Laboratory animals.

### 2.2.2 Drugs

(-)-Nicotine hydrogen tartrate salt [(-)-1-methyl-2-(3-pyridyl) pyrrolidine (-)-bitartrate salt] was purchased from Sigma-Aldrich (St. Louis, MO). MLA and dihydro- $\beta$ -erythroidine (DH $\beta$ E) were purchased from RBI (Natick, MA). Naloxone hydrochloride dehydrate, PNU 120596 [1-(5-Chloro-2,4-dimethoxy-phenyl)-3-(5-methyl-isoxazol-3-yl)] and PHA-543613 [N-[(3R)-1-azabicyclo[2.2.2]oct-3-yl]furo[2,3-c]pyridine-5-carboxamide] was obtained from the Drug Supply Program of the National Institute on Drug Abuse (Rockville, MD). NS 1738 [N-(5-Chloro-2-hydroxyphenyl)-N'-[2-chloro-5-(trifluoromethyl)phenyl]] was purchased from Tocris Biosciences (Minneapolis, MN). U0126 [1,4-Diamino-2,3-dicyano-1,4-bis(o-aminophenylmercapto)butadiene monoethanolate] was purchased from Cell Signaling Technology (Danvers, MA). All drugs except for PNU 120596, NS 1738 and U0126 were dissolved in physiological saline (0.9% sodium chloride) and injected subcutaneously at a total volume of 1ml/100 g body weight unless noted otherwise. PNU 120596, NS1738 and U0126 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] and administered intraperitoneal (i.p.). All doses are expressed as the free base of the drug.

### 2.2.3 Antinociceptive Tests

#### 2.2.3.1 *Tail-Flick Test*

The antinociceptive effect of drugs was assessed by the tail-flick method of D'Amour and Smith (1941), as modified by Dewey et al. (1970). A control response (2–4 s latency) was determined for each mouse before treatment, and test latency was determined after drug administration. To minimize tissue damage, a maximum latency of 10 s was imposed. Antinociceptive response was calculated as percent maximum possible effect (%MPE), where  $\%MPE = [(test\ value - control\ value) / (cut-off\ (10\ s) - control\ value)] \times 100$ . Groups of 6 to 8 animals were used for each dose and for each treatment. For the tail-flick test, mice were given a 5 min pretreatment with either vehicle or nicotine (2.5mg/kg s.c.) or a 15 min pretreatment of PHA-543613 (8mg/kg s.c.), PNU-120596 (4 and 8 mg/kg i.p.), or NS1738 (10 and 30 mg/kg i.p.).

#### 2.2.3.2 Hot-Plate Test

Mice were placed into a 10-cm wide glass cylinder on a hot-plate (Thermojust Apparatus, Columbus, OH) as a measure of supraspinal antinociception. The hot plate is a rectangular heated surface surrounded by plexiglass and maintained at 55°C. The device is connected to a manually operated timer that records the amount of time the mouse spends on the heated surface before showing signs of nociception (e.g. jumping, paw licks). Two control latencies at least 10 min apart were determined for each mouse. The normal latency (reaction time) was 8 to 12 s was assessed with a saline injection. To avoid tissue damage, the hot-plate will automatically disengage after 40 seconds. Antinociceptive response was calculated as % MPE, where  $\%MPE = [(test\ value - control) / (cut-off\ time\ (40\ s) - control) \times 100]$ . The reaction time was scored when the animal jumped or licked its paws. Mice were tested at different times after i.p. injection of NS1738 and PNU-120596. For the hot-plate test, mice were given a 5 min

pretreatment with either vehicle or nicotine (2.5mg/kg s.c.) or a 15 min pretreatment of PHA-543613 (8mg/kg s.c.), PNU-120596 (4 and 8 mg/kg i.p.), or NS1738 (10 and 30 mg/kg i.p.).

### 2.2.3.3 *Mechanical Sensitivity Test*

Mechanical sensitivity was determined according to the method of Chaplan et al. (1994). Mice were placed in a Plexiglas cage with mesh metal flooring and allowed to acclimate for 30 min before testing. A series of calibrated von Frey hairs (Stoelting, Wood Dale, IL) with logarithmically incremental stiffness ranging from 2.83 to 5.88 expressed dsLog10 of [10 £ force in (mg)] were applied to the paw with a modified up-down method (Dixon, 1965). In the absence of a paw withdrawal response to the initially selected hair, a thicker hair corresponding to a stronger stimulus was presented. In the event of paw withdrawal, the next weaker stimulus was chosen. Each hair was presented perpendicularly against the paw, with sufficient force to cause slight bending, and held 2–3 s. The stimulation of the same intensity was applied 5 times to the hind paw at intervals of a few seconds. The mechanical threshold was expressed as force in (g), indicating the force of the Von Frey hair to which the animal reacted (paw withdrawn, licking or shaking). The mechanical allodynia thresholds were measured before (pre-drug) and at 30 min after i.p treatment of the following drugs: PNU 120596 and NS1738.

### 2.2.3.4 *Formalin Test*

The formalin test was carried out in an open Plexiglas cage, with a mirror placed under the floor to allow an unobstructed view of the paws. Mice were allowed to acclimate for 15 min in the test cage before formalin injection. Each animal was injected with 20 µl of 2.5% formalin in the intraplantar (i.pl.) region of the right hindpaw. Mice were then observed (two at a time) 0-5 min (Phase 1) and 20-45 min (Phase 2) post-formalin, and the amount of time spent licking the



injected paw was recorded. The period between the two phases of nociceptive responding is generally considered to be a phase of weak activity. The amount of time spent licking the injected paw was recorded with a digital stopwatch. The Formalin test was carried out in an open Plexiglas cage, with a mirror placed at a 45-degree angle behind the cage to allow an unobstructed view of the paws.

PNU-120596, NS1738, or vehicle were injected i.p. at different times before the formalin injection. For the antagonists studies, MLA, DHBE or naloxone was injected s.c. 5 min before the formalin injection. Site studies were carried out by pretreating the mice with PNU-120596 (4mg/kg i.p.) 15 min before i.t. (10 $\mu$ g/mouse) or i.pl. (10 $\mu$ g/5 $\mu$ l contralateral paw or ipsilateral paw) MLA.

#### 2.2.4 Intrathecal Injections

I.t. injections were performed free-hand using a 30-gauge needle attached to a glass microsyringe inserted between the L5 and L6 lumbar space in unanesthetized mice according to the method of Hylden and Wilcox (1980). The injection volume in all cases was 5  $\mu$ l. The accurate placement of the needle was evidenced by a reflexive “flick” of the mouse's tail.

#### 2.2.5 Locomotor Activity Test

Mice were placed into individual Omnitech photocell activity cages (28 x 16.5 cm) after 15 minutes after the i.p. administration of either i.p. vehicle or PNU-120596 at different doses. Interruptions of the photocell beams (two banks of eight cells each), which assess walking and rearing, were then recorded for the next 30 minutes. Data were expressed as the number of photocell interruptions.

### 2.2.6 Motor Coordination Test

In order to measure motor coordination, we used Rotarod (IITC Inc. Life Science). The animals are placed on textured drums (1¼ inch diameter) to avoid slipping. When an animal drops onto the individual sensing platforms, test results are recorded. Five mice tested at a rate of 4 rpm. Naive mice were trained until they could remain on the rotarod for 5 min. Animals that failed to meet this criterion within three trials were discarded. Fifteen minutes after the injection of vehicle or drugs, mice were placed on the rotarod for 5 min. If a mouse fell from the rotarod during this time period, it was scored as motor impaired. Percent impairment was calculated as follows: % impairment =  $[(180 - \text{test time} / 180) * 100]$ . Mice were pretreated with either i.p. vehicle or PNU-120596 at different doses 15 min before the test.

### 2.2.7 Chronic PNU-120596 Administration Protocol

Mice were administered PNU-120596 (4 mg/kg, i.p.) once a day for six days and then were challenged with PNU-120596 (4mg/kg, i.p.) on day 7 and evaluated in the formalin test. Another group was exposed to vehicle for six days and then challenged with PNU-120596 on the seventh day. A vehicle-control group, in which mice were exposed to seven days of vehicle, was also included.

### 2.2.8 Time Course of PNU-120596 & NS1738 Levels in Brain and Plasma

For the determination of plasma and brain concentrations of the parent compound, naïve mice were dosed with the compounds as indicated and sacrificed at various time points post-dosing. For analytical determination of plasma concentrations, blood was collected into heparinized tubes and then centrifuged, and the separated plasma was frozen at -20°C until analysis. For the determination of brain concentrations, animals were decapitated at the various

time points, and the brains were immediately removed and rapidly freed from blood vessels as much as possible. The resulting brain tissues were immediately frozen at  $-20^{\circ}\text{C}$ , weighed and homogenized, and then the homogenate was stored at  $-20^{\circ}\text{C}$ . For analysis, compounds were extracted from the samples via liquid-liquid extraction and were quantified by liquid chromatography/mass spectroscopy. As a result, it is able to measure concentrations of either NS-1738 or PNU-120596 down to 1 ng/ml in plasma ( $\sim 3\text{nM}$ ) and 10 ng/ml in brain ( $\sim 30\text{nM}$ ) (Timmermann et al 2007). Brain and plasma levels of NS1738 and PNU-120596 were obtained by Abbott laboratories.

### 2.2.9 Statistical Analysis

The data obtained were analysed using GraphPad software programme and expressed as the mean  $\pm$  S.E.M. Statistical analysis was done by one-way or two-way analysis of variance test ANOVA followed by post hoc, Tukey or Bonferroni tests. *P*-values less than 0.05 ( $P < 0.05$ ) were considered significant.

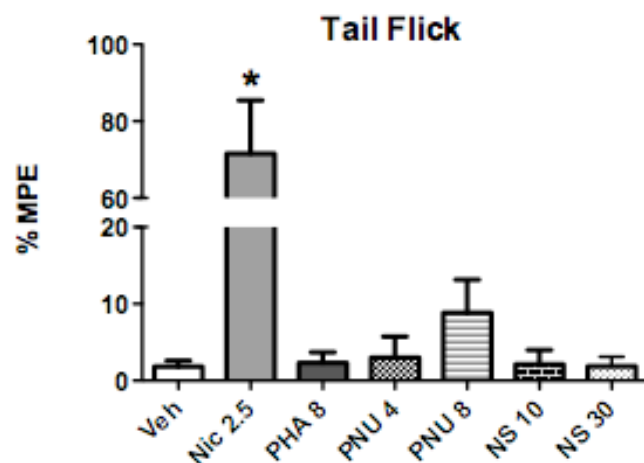
## 2.3 Results

### 2.3.1 Lack of Antinociceptive Effect of $\alpha 7$ PAMs in Acute Thermal Pain in Mice

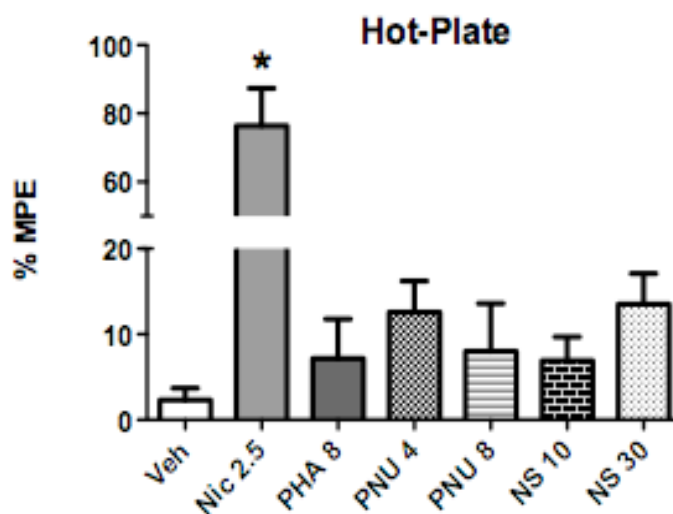
The tail-flick and hot-plate tests were used to determine the antinociceptive effects of the  $\alpha 7$  agonist and  $\alpha 7$  PAMs after i.p. administration in acute thermal pain models. A 5 min pretreatment of nicotine (2.5 mg/kg) induced significant antinociceptive effects in the tail-flick [ $F_{(6,35)} = 20.91$ ,  $p < 0.0001$ ] and hot-plate [ $F_{(6,35)} = 22.68$ ,  $p < 0.0001$ ] tests when compared to vehicle (Figure 1A & B). In contrast, none of the  $\alpha 7$  compounds showed significant antinociceptive effects at any doses tested in the tail-flick [ $F_{(5,30)} = 1.330$ ,  $p = 0.2783$ ] or hot-plate [ $F_{(5,30)} = 1.154$ ,  $p = 0.3546$ ] tests. Figure 1 (A & B) shows the lack of antinociceptive

activity 15 min after administration of the PNU-120596 (4 and 8 mg/kg, i.p.), NS1738 (10 and 30 mg/kg, i.p.) and PHA-543613 (8 mg/kg, s.c.) in both tests. A similar lack of effect was observed at later pretreatment times (1 and 3 hr) after injection (Data not shown).

(A)



(B)



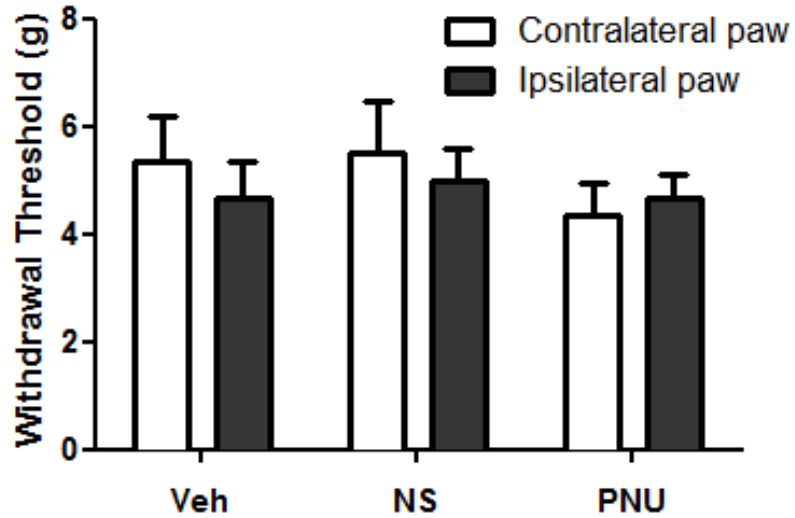
**Figure 1. Antinociceptive effect of various  $\alpha 7$  nicotinic compounds in the tail-flick and hot-plate tests after acute administration in mice.**

Figure (A) Effects of nicotine (2.5 mg/kg, s.c.), PHA543613 (8mg/kg., s.c.), PNU-120596 (4 and 8 mg/kg., i.p.) and NS1738 (10 and 30mg/kg., i.p.) in the tail-flick test. Mice were pretreated s.c. with nicotine 5 min before testing. The other treatment groups received PHA-543613, PNU-120596 or NS1738 15 min before the tail-flick test. Figure (B) Effects of nicotine, PHA-543613, PNU-120596, and NS1738 in the hot-plate test. Mice were treated with the same doses and pretreatment time as in the tail-flick test. Each group represents the mean  $\pm$  SE of 6-8 mice and \*denotes  $p < 0.05$  vs. vehicle. Each point represents the mean  $\pm$  SE of 6 mice. NS = NS1738; PNU = PNU-120596; Nic = nicotine; Veh = vehicle; %MPE = percent of maximum effect

### 2.3.2 The Effects of $\alpha 7$ PAMs on Mechanical Sensitivity

The effects of PNU-120596 (8 mg/kg, i.p.) and NS1738 (30 mg/kg, i.p.) on the mechanical sensitivity were measured using calibrated von Frey filaments 15 min after injection. As shown in Figure 2, neither PNU-120596 nor NS1738 produced significant [ $F_{2(2,3)} = 2.172$ ,  $p = 0.2610$ ] differences in mechanical sensitivity in response to hind paw stimulation using von Frey filaments.

(A)



**Figure 2. The effects of  $\alpha 7$  PAMs on mechanical sensitivity.**

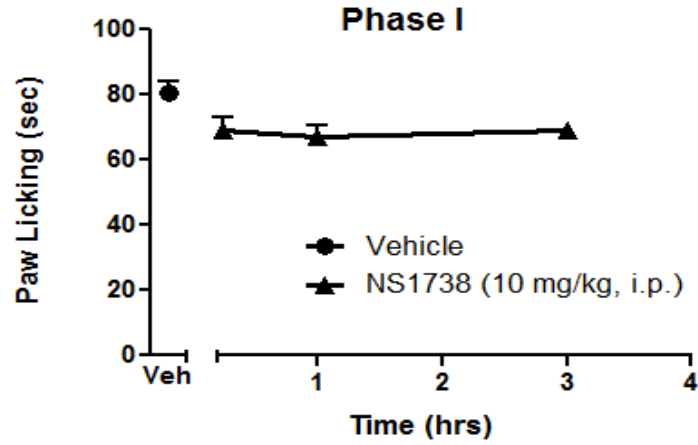
Effects of NS1738 (30 mg/kg, i.p.) and PNU-120596 (4 mg/kg, i.p.) on mechanical sensitivity in mice. The two  $\alpha 7$  PAMs were given i.p to animals and 30 min later, their withdrawal thresholds (g) were measured. Each point represents the mean  $\pm$  SE of 6 mice. NS = NS1738; PNU = PNU-120596; Veh = vehicle.

### 2.3.3 Time Course of Type I $\alpha$ 7 PAM in the Formalin Test

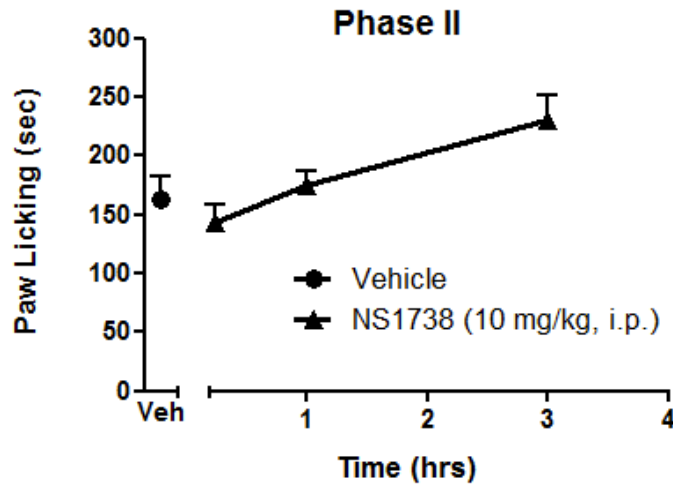
NS1738, a type I  $\alpha$ 7 PAM, was evaluated for its effect in the formalin test, a mouse model of tonic pain. We first tested NS1738 in the formalin test at different times after injection. As shown in Figure 3 (A & B), NS1738 (10mg/kg, i.p.) did not produce a significant decrease [ $F_{(3,35)} = 2.108, p = 0.1169$ ] in the duration of i.pl. formalin-induced nociceptive behavior in phase I and II at different times (15 min and 1 and 3 hr) after injection.



(A)



(B)

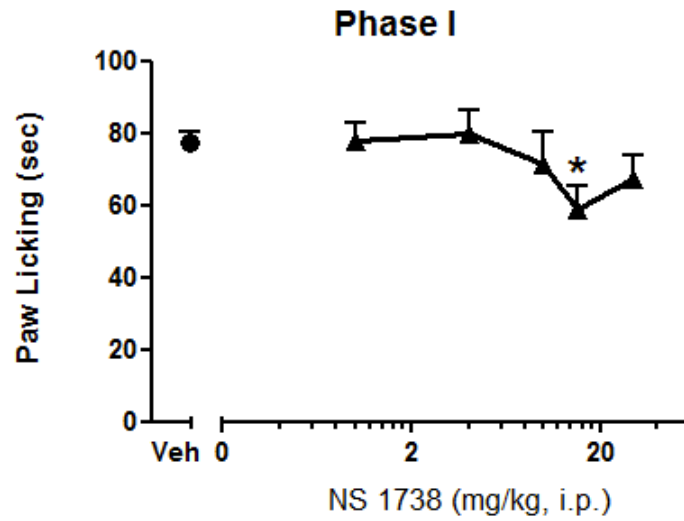


**Figure 3. Effects of  $\alpha 7$  type I PAM NS1738 in the mouse formalin test of persistent pain.** Time-course of the effects of NS1738 on (A) phase I and (B) phase II in the formalin test after i.p. administration. Mice treated with either vehicle or NS1738 (10 mg/kg, i.p) at different times after injection (15min, 1h and 3h) before i.pl. formalin injection in the right paw. Each point represents the mean  $\pm$  S.E.M of total time spent licking for 8-10 mice per group and \*denotes  $p < 0.05$  vs. vehicle. Veh = vehicle.

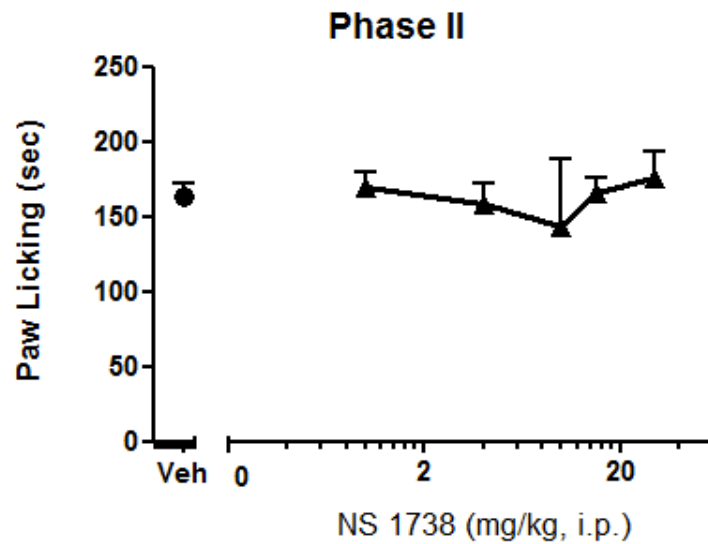
#### 2.3.4 Dose-Response Curve of Type I $\alpha$ 7 PAM in the Formalin Test

We then tested several NS1738 doses (1, 4, 10, 15 and 30 mg/kg, i.p.) 15 min after injection in the formalin test. As shown in Figure 4A, NS1738 produces a small but significant decrease  $F_{5,37} = 7.660$ ,  $p < 0.0001$  of formalin-induced nociceptive behavior at the two highest doses of 15 and 30 mg/kg (-30% decrease compared to vehicle). However, NS1738 failed to elicit a significant decrease [ $F_{(3,33)} = 1.450$ ,  $p = 0.2326$ ] in the nociceptive effects of phase II at any of the doses tested when compared to vehicle (Figure 4B).

(A)



(B)

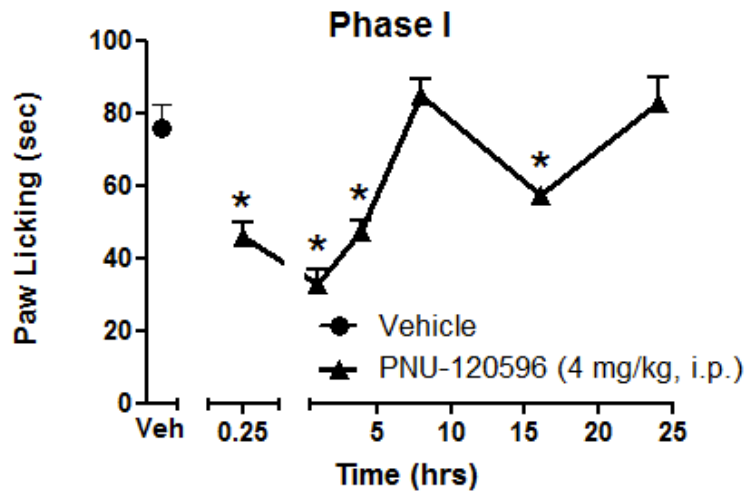


**Figure 4. Effects of  $\alpha 7$  type I PAM NS1738 in the mouse formalin test of persistent pain.** Dose-response relationship for NS1738 was then established in mice 15 min after i.p. injection of various doses of drugs in (A) phase I and (B) phase II of the formalin test. Each point represents the mean  $\pm$  S.E.M of total time spent licking for 8-10 mice per group and \*denotes  $p < 0.05$  vs. vehicle. Veh = vehicle.

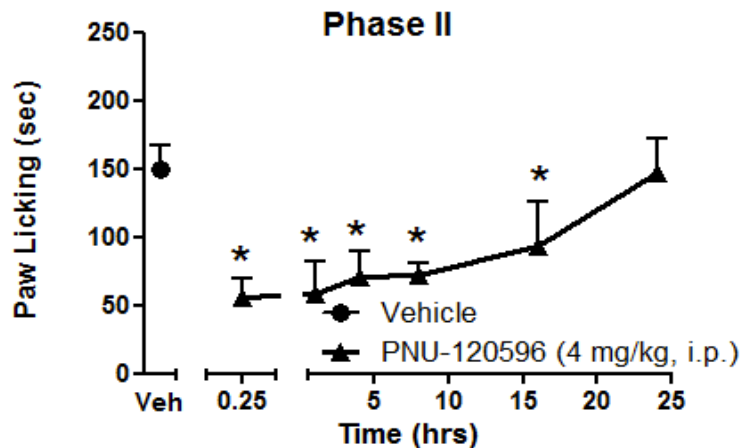
### 2.3.5 Time Course of Type II $\alpha$ 7 PAM in the Formalin Test

Type II  $\alpha$ 7 PAM, PNU-120596, was evaluated for its effect in the formalin test. We first determined the time-course of PNU-120596 effects after injection. In contrast to NS1738, PNU-120596 significantly decreased the spontaneous pain-related (nociceptive) behavioral response to formalin. As shown in Figure 5 (A & B), the onset of action for PNU-120596 (4 mg/kg, i.p.) was fairly rapid with maximum antinociception occurring between 15 and 60 min. However, the duration of PNU-120596-induced antinociception was long. Indeed, PNU-120596's effect disappeared completely within 24 hr after i.p. administration of the drug in mice. As illustrated in Figure 5B, PNU-120596's effect in phase II gradually diminished to 40% decrease at 16 hr (significantly different from vehicle) [ $F_{(6,51)} = 22.99, p < 0.0001$ ] and disappeared completely within 24 hr after injection. A similar time-course [ $F_{(6,51)} = 30.78, p < 0.0001$ ] was seen in phase I with a rebound at time 8 hr (Figure 5A).

(A)



(B)



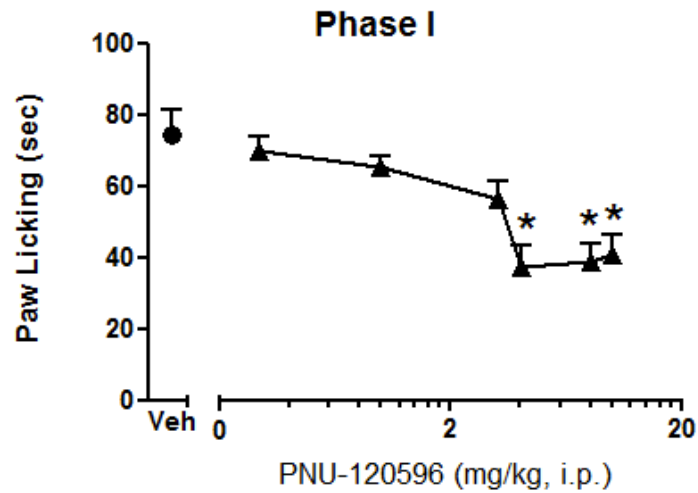
**Figure 5. Effects of  $\alpha 7$  type II PAM PNU-120596 in the mouse formalin test of persistent pain.**

Time-course of the effects of PNU-120596 on (A) phase I and (B) phase II in the formalin test after i.p. administration. Mice treated with either vehicle or PNU-120596 (4 mg/kg, i.p.) at different times after injection (15min, 1, 3, 6, 16 and 24 hrs) before i.pl. formalin injection in the right paw. Each point represents the mean  $\pm$  S.E.M of total time spent licking for 8-10 mice per group and \*denotes  $p < 0.05$  vs. vehicle. Veh = vehicle.

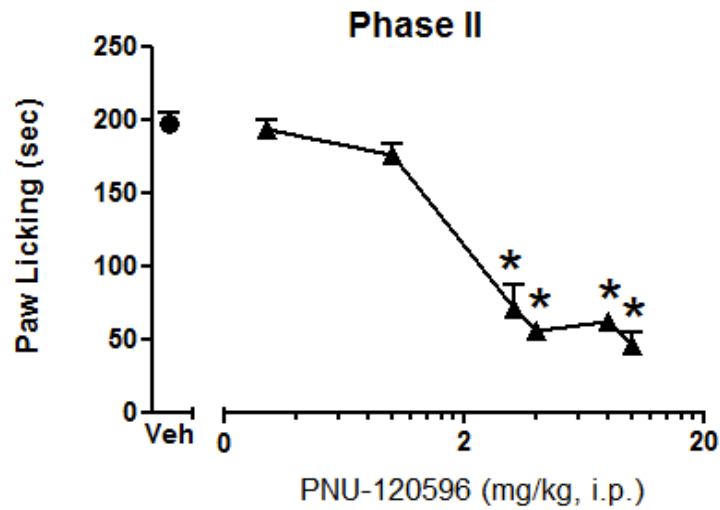
### 2.3.6 Dose-Response Curve of Type II $\alpha$ 7 PAM in the Formalin Test

Dose-response relationship was then established for PNU-120596 in mice by measuring antinociception at the time of maximal effect of 15 min (Figure 6A & B). PNU-120596 produced a dose-responsive decrease in formalin nociceptive behavior with an ED<sub>50</sub> ( $\pm$ CL) of 12.8 (6.9-23.3) and 2.78 (2.3-3.3) mg/kg in phases I and II respectively. The dose of 4 mg/kg of PNU-120596 was used for the subsequent studies.

(A)



(B)



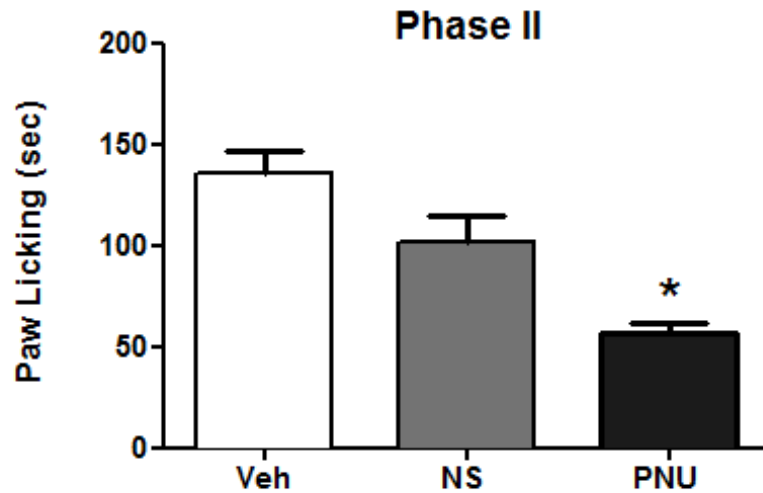
**Figure 6. Effects of  $\alpha 7$  type II PAM PNU-120596 in the mouse formalin test of persistent pain.**

Dose-response relationship for PNU-120596 was then established in mice 15 min after i.p. injection of various doses of drugs in (A) phase I and (B) phase II of the formalin test. Each point represents the mean  $\pm$  S.E.M of total time spent licking for 8-10 mice per group and \*denotes  $p < 0.05$  vs. vehicle. Veh = vehicle.

### 2.3.7 Effects of Type I and II $\alpha 7$ PAMs after i.t. Administration in the Formalin Test

The lack of effect of NS1738 after peripheral administration (i.p.) prompted us to give the drug centrally via the intrathecal route. Mice were pretreated i.t. with NS1738 at the dose of 15 $\mu$ g/mice and tested 5 min later in the formalin test. As shown Figure 7 the type I PAM elicited no significant effect in decreasing nociceptive behavior compared to vehicle-treated animals. However, an i.t. administration of type II PAM PNU-120596 produced a significant [ $F_{(2,18)} = 14.64, p = 0.0002$ ] antinociceptive effect in phase II of the formalin test in mice at a dose 7-times lower (2  $\mu$ g/mice) than NS1738.



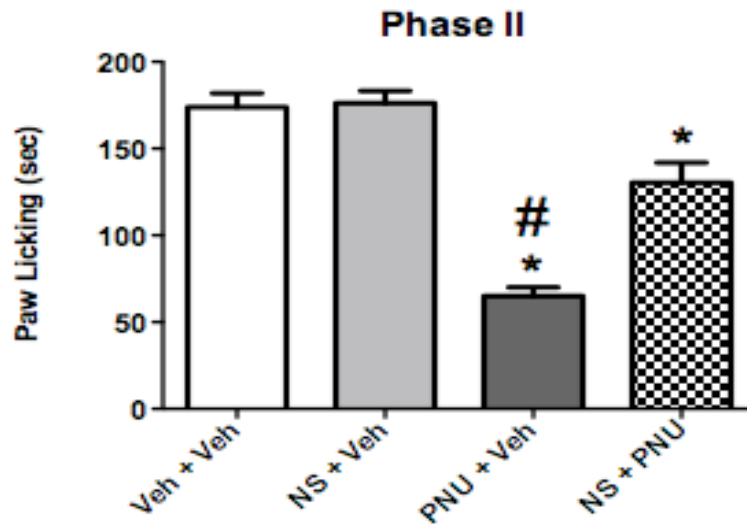


**Figure 7. Antinociceptive effect of NS1738 and PNU-120596 in the formalin test after i.t. administration.**

Mice were pretreated i.t. with either vehicle, NS1738 (15  $\mu\text{g}/\text{mice}$ ) or PNU-120596 (2  $\mu\text{g}/\text{mice}$ ) and tested 5 min in the formalin model. While NS1738, a type I PAM elicited no significant effect in decreasing nociceptive behavior in phase II of the formalin test. Each point represents the mean  $\pm$  S.E.M of total time spent licking for 8-10 mice per group and \*denotes  $p < 0.05$  vs. vehicle. NS = NS1738; PNU = PNU-120596; Veh = vehicle.

### 2.3.8 NS1738 Blocks PNU-120596's Antinociceptive Effect in the Formalin Test

While NS1738 failed to elicit antinociceptive effects after peripheral and central administration, it blocked the actions of PNU-120596 in the formalin test. Indeed, pretreatment of mice with NS1738 (30mg/kg, i.p.) partially blocked the antinociceptive response [ $F_{(3,23)} = 24.14, p < 0.0001$ ] elicited by an active dose of PNU-120596 (4 mg/kg, i.p.) during phase II of the formalin test (Figure 8).



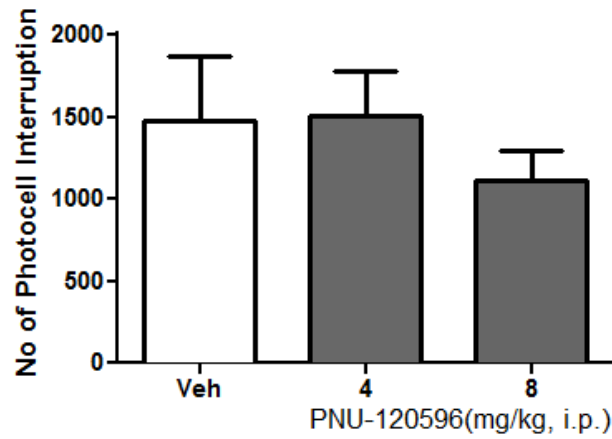
**Figure 8. NS1738 blocks PNU-120596's antinociceptive effect in the formalin test.**

Mice were pretreated with NS1738 (30mg/kg, i.p.) 15 min before an active dose of PNU-120596 (4mg/kg, i.p.) and tested 15 min after the second injection in the formalin test. The total time spent licking the right hind paw was measured in the late phase. Each bar represents the mean  $\pm$  S.E.M for each group of 6-8 mice. The asterisks denote the significance levels when compared with the control group: \* $p < 0.05$  and # $p < 0.05$  versus NS + PNU. NS = NS1738; PNU = PNU-120596; Veh = vehicle.

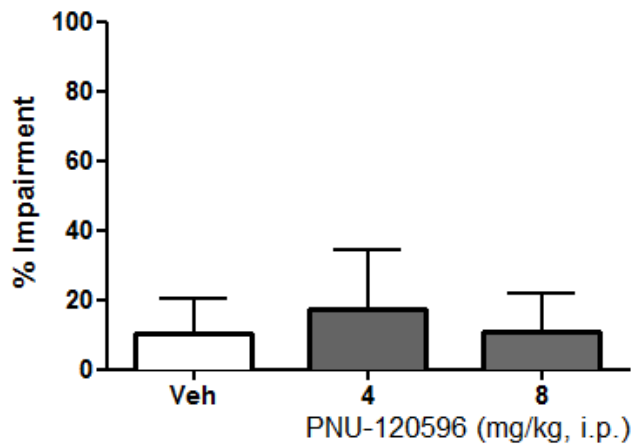
### 2.3.9 The Lack of Effect of $\alpha 7$ type II PAM on Locomotor Activity and Coordination of Mice

In order to determine if the effects of PNU-120596 in the formalin test are not due to disruption of the locomotor activity during testing, we evaluated the effect of antinociceptive doses of PNU-120596 on spontaneous activity and motor coordination of mice. As seen in Figure 9, mice treated with PNU-120596 at doses of 4 and 8 mg/kg, i.p. did not show significant changes in locomotor activity (locomotor test) [ $F_{(2,11)} = 0.8252$ ,  $p = 0.4365$ ] or motor coordination (rotarod test) [ $F_{(2,12)} = 0.08455$ ,  $p = 0.9195$ ], 15 min after testing.

(A)



(B)

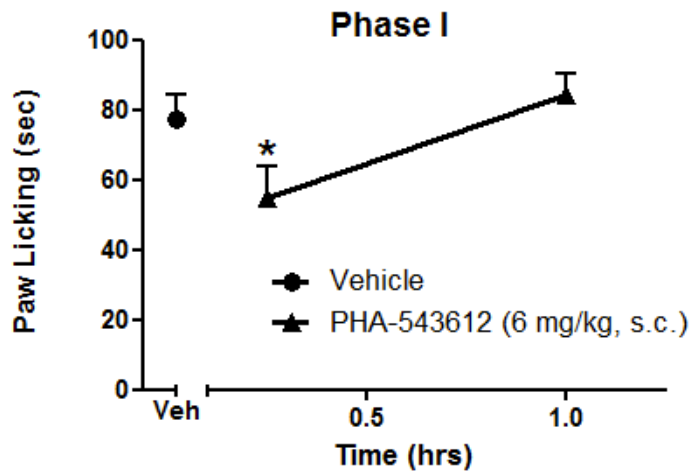


**Figure 9. Effects of PNU-120596 on locomotor activity and motor coordination of mice.** (A) The effects of vehicle and PNU-120596 (4 and 8 mg/kg, i.p.) on mouse locomotor activity. Animals were tested 15 min after injection and their locomotor activity were measured for 30 min. (B) The effects of vehicle and PNU-120596 (4 and 8 mg/kg, i.p.) in the rotarod test after administration in mice. Each point represents 6-8 animals per group.

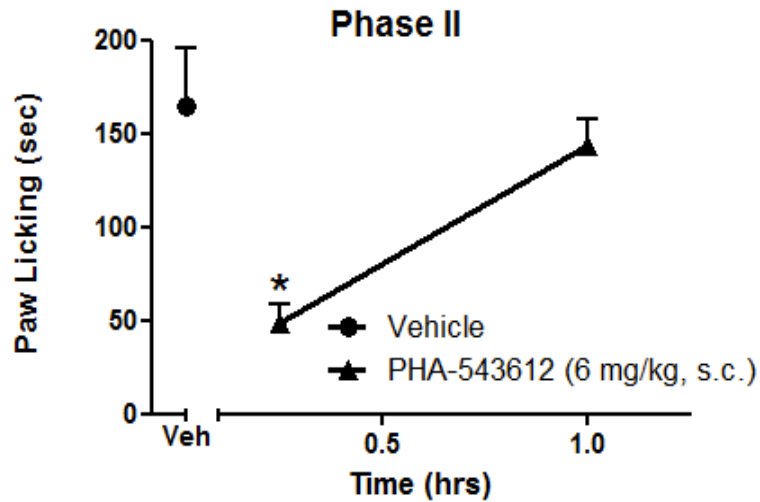
### 2.3.10 Time Course of a Selective $\alpha 7$ Agonist in the Formalin Test

We compared the effects of the type II  $\alpha 7$  PAM, PNU-120596 in the formalin test to a full  $\alpha 7$  agonist, PHA-543613. PHA-543613 at 6mg/kg given s.c. significantly reduced the formalin-induced nociceptive behavior during both early  $F_{2,15} = 4.366$ ,  $p = 0.0320$  and late  $F_{2,15} = 25.80$ ,  $p < 0.0001$  phases (Figure 10A & B). The onset of action was relatively fast with a maximum between 0 and 15 min and the effects disappeared within 60 min after the injection (Figure 10A & B).

(A)



(B)



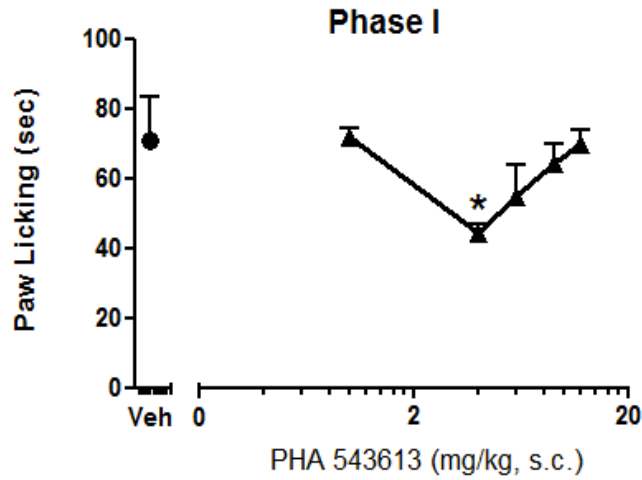
**Figure 10. Effects of  $\alpha 7$  agonist PHA-543613 in the mouse formalin test of persistent pain.** Time-course of the effects of PHA-543613 on (A) phase I and (B) phase II in the formalin test after s.c. administration. Mice treated with either vehicle or PHA-543613 (6 mg/kg, s.c.) at 15 and 60 min after injection before i.pl. formalin injection in the right paw. Each point represents the mean  $\pm$  S.E.M of total time spent licking for 8-10 mice per group and \*denotes  $p < 0.05$  vs. vehicle. Veh = vehicle

### 2.3.11 Dose-Response Curve of a Selective $\alpha 7$ Agonist in the Formalin Test

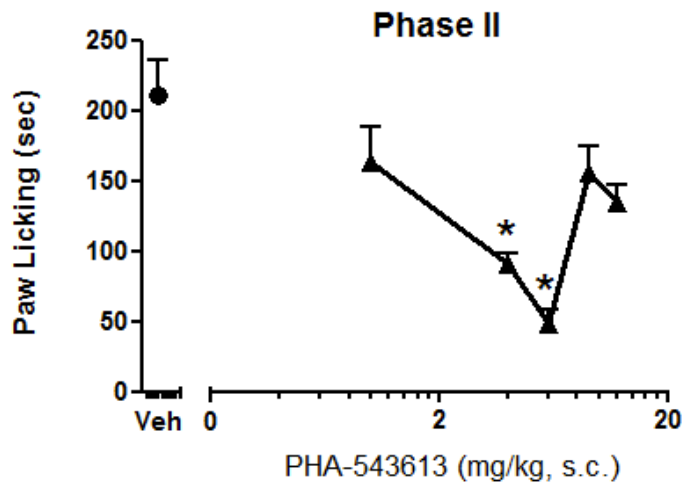
When a dose-response relationship was established, a U-shape curve emerged for both phase I and II (Figure 11A & B). PHA-543613 reduced formalin nociceptive behaviors at narrow range of doses (4 and 6 mg/kg in phase II) but the antinociception effect of the drug was lost at higher doses.



(A)



(B)

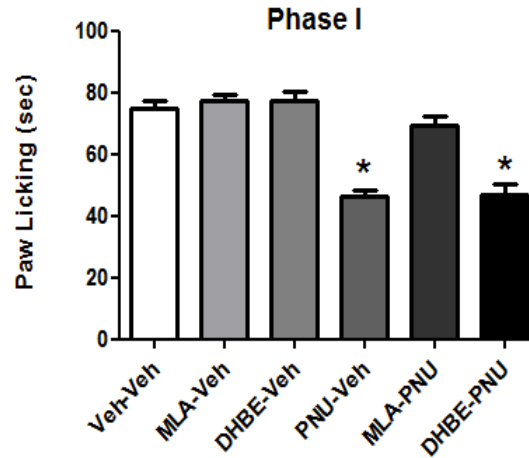


**Figure 11. Effects of  $\alpha 7$  agonist PHA-543613 in the mouse formalin test of persistent pain.** Dose-response relationship for PHA-543613 was then established in mice 15 min after s.c. injection of various doses of drugs in (A) phase I and (B) phase II of the formalin test. Each point represents the mean  $\pm$  S.E.M of total time spent licking for 8-10 mice per group and \*denotes  $p < 0.05$  vs. vehicle. Veh = vehicle

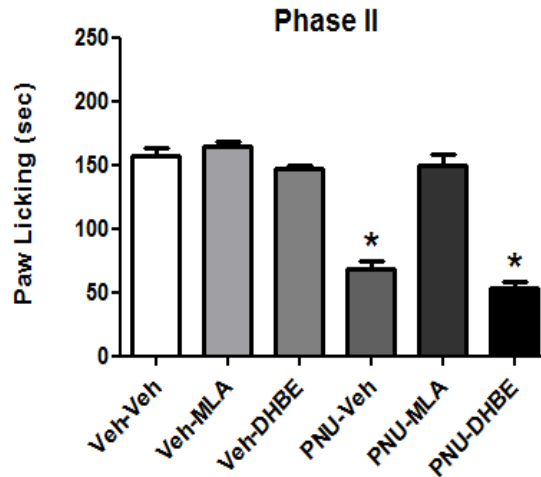
### 2.3.12 Role of $\alpha 7$ and $\beta 2^*$ nAChRs Subtypes in PNU-120596-induced Antinociception in the Formalin Test

We examined the role of  $\beta 2^*$  and  $\alpha 7$  nAChRs subtypes in mediating the antinociceptive effect of PNU-120596 (4 mg/kg i.p.). As predicted, the  $\alpha 7$  antagonist MLA (10 mg/kg, s.c.) completely blocked PNU-120596's effects in both phase I [ $F_{(5,34)} = 30.77$ ,  $p < 0.0001$ ] and II [ $F_{(5,34)} = 63.11$ ,  $p < 0.0001$ ] (Figure 12A & B). In contrast, DH $\beta$ E (2 mg/kg, s.c.), a  $\beta 2$ -containing selective antagonist, failed to block PNU-120596's actions in the formalin test (Figure 12A & B).

(A)



(B)



**Figure 12. Nicotinic receptors subtypes involved in PNU-120596-induced antinociception in the formalin test.**

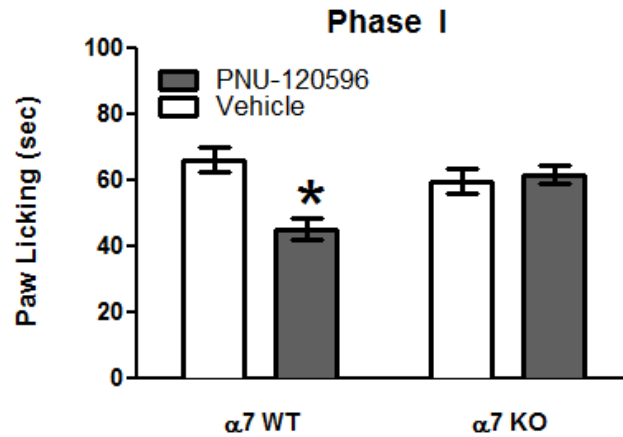
Blockade of the antinociceptive effect of PNU-120596 in the (A) phase I and (B) phase II of formalin test by different nicotinic antagonists. Mice were pretreated with MLA (10 mg/kg, s.c.) or DH $\beta$ E (2 mg/kg, s.c.) 15 min before an active dose of 4 mg/kg of PNU-120596. Fifteen min later, mice were injected with formalin (2.5% intraplantary, 20  $\mu$ l) and then observed for pain behaviors. Each bar represents the mean  $\pm$  S.E.M for each group of 6-8 mice and \*denotes  $p < 0.05$  vs. vehicle-vehicle group. Veh = vehicle; PNU = PNU-120596.

### 2.3.13 Role of $\alpha 7$ nAChRs Subtypes in PNU-120596-induced Antinociception in the Formalin

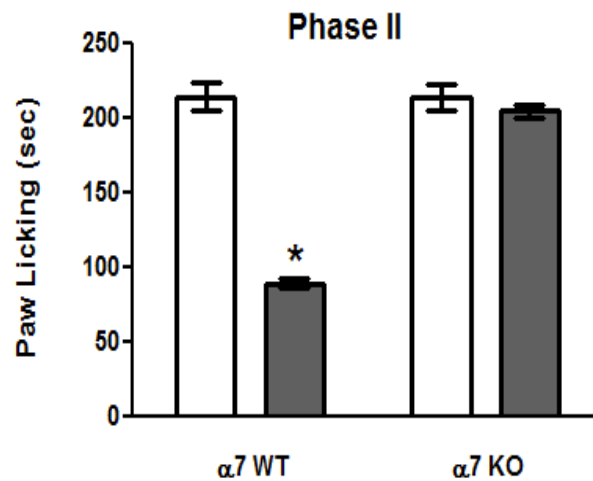
#### Test

The blockade of PNU-120596's effects in the formalin test being mediated through  $\alpha 7$  nAChRs was confirmed using the  $\alpha 7$  KO mice. As shown in Figure 13 (A & B), PNU-120596-induced antinociception in both phase I [treatment:  $[F_{(1,9)} = 8.136, p = 0.0106]$ ; gene:  $[F_{(1,9)} = 2.197, p = 0.1556]$ ; interaction:  $[F_{(1,18)} = 11.56, p = 0.0032]$  and II [treatment:  $[F_{(1,9)} = 107.3, p < 0.0001]$ ; gene:  $[F_{(1,9)} = 79.20, p < 0.0001]$ ; interaction:  $[F_{(1,18)} = 79.20, p < 0.0001]$  was lost in the  $\alpha 7$  KO mice compared to their WT littermates (Figure 13A & B).

(A)



(B)



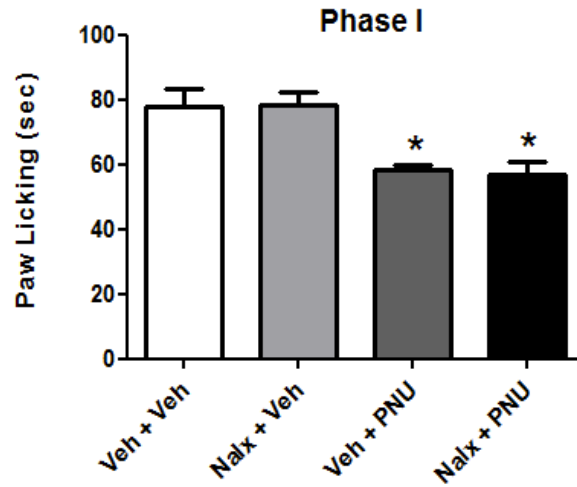
**Figure 13. Antinociceptive effects of PNU-120596 in the  $\alpha 7$  WT and KO mice using the formalin Test.**

Mice received a dose of 4 mg/kg, i.p. of PNU-120596 in  $\alpha 7$  WT and KO mice and 15 min later were tested in (A) the phase I and (B) phase II of formalin test. Data was expressed as mean  $\pm$  SEM of licking time. Each group represents the mean  $\pm$  SE of 6-8 mice and \*denotes p < 0.05 vs. vehicle.

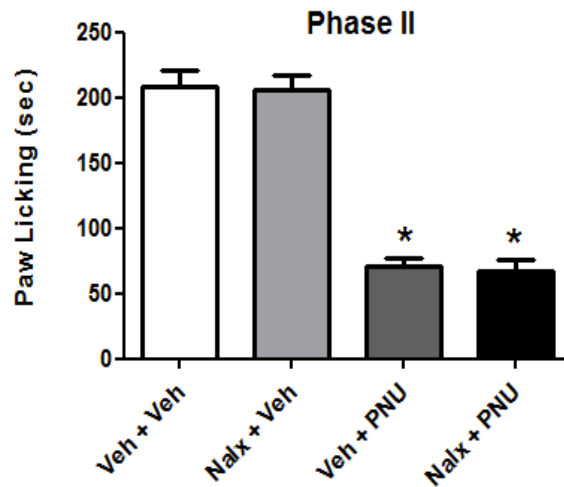
#### 2.3.14 Role of $\mu$ opioid Receptors in PNU-120596-induced Antinociception in the Formalin Test

Pretreatment with naloxone (1 mg/kg, s.c.), an opioid receptor antagonist, did not abolish the antinociceptive effect of PNU-120596 given at the dose of 4 mg/kg (i.p.) in phase I [ $F_{(3,16)} = 9.237$ ,  $p = 0.0009$ ] and phase II [ $F_{(3,16)} = 69.05$ ,  $p < 0.0001$ ] (Figure 14A &B).

(A)



(B)



**Figure 14. Lack of blockade of PNU-120596-induced antinociception in the formalin test by naloxone.**

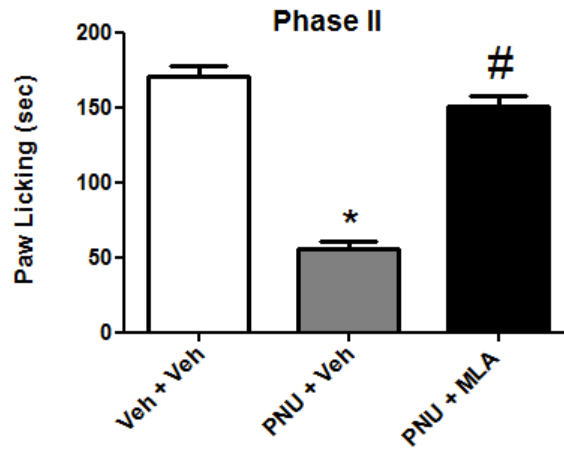
Mice were pretreated with s.c. naloxone 15 min prior to PNU-120596 (4mg/kg, i.p.) injection. They were tested 15 min after the second injection in the formalin test. The time spent licking the injected paw was recorded in (A) the early and (B) late phase after the formalin injection. Data was expressed as mean  $\pm$  SEM of licking time. Each group represents the mean  $\pm$  SE of 6-8 mice and \*denotes  $p < 0.05$  vs. vehicle-vehicle group.

### 2.3.15 Contribution of Peripheral and Spinal $\alpha 7$ nAChRs to PNU-120596's Antinociceptive Response

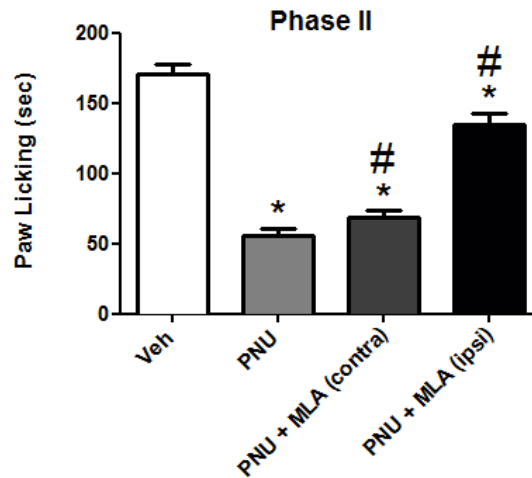
Since  $\alpha 7$  nAChRs subtypes are present in both the periphery and the spinal cord, we examined the contribution of these sites to PNU-120596's antinociceptive response in the formalin test. When the  $\alpha 7$  antagonist MLA was given i.t. (10  $\mu\text{g}/\text{mouse}$ ) 5 min before PNU-120596 (4mg/kg, i.p.) administration, the effect of the  $\alpha 7$  PAM II was completely reversed [ $F_{(2,18)} = 17.33, p < 0.0001$ ] (Fig. 15A). Local i.pl. administration of MLA (10  $\mu\text{g}/\text{mouse}$ ) partially blocked [ $F_{(3,23)} = 72.37, p < 0.0001$ ] PNU-120596-induced antinociception. In contrast, the contralateral paw over the time period observed MLA given i.pl. to the left paw failed to block the antinociceptive effect of PNU-120596 (Fig. 15B).



(A)



(B)

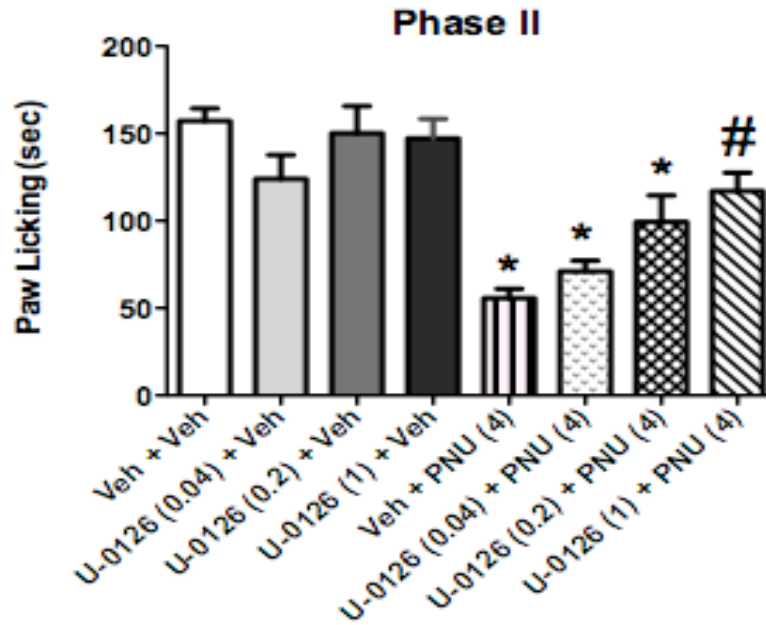


**Figure 15. Blockade of PNU-120596's antinociceptive effect after i.t. or i.pl. MLA administration in the formalin test.**

(A) The effects of i.t. MLA on PNU-120596's antinociceptive effect in the phase II of the formalin test. Mice were pretreated with either PNU-120596 (4mg/kg, i.p.) or vehicle (i.p.) 15 min prior to MLA (10µg/5µl i.t.) injection, and tested 5 min after later in the formalin test. (B) The effects of i.pl. MLA on PNU-120596's antinociceptive effect in the phase II of the formalin test. Mice were pretreated with either PNU-120596 (4mg/kg, i.p.) or vehicle (i.p.) 15 min prior to i.pl. MLA (10µg/5µl in the contralateral or ipsilateral paw) injection, and tested 5 min after later in the formalin test. Each bar represents the mean ± S.E.M for each group of 6-8 mice. \*p < 0.05 versus vehicle-vehicle group; #p < 0.05 versus 4mg/kg PNU-120596. Veh = vehicle; PNU = PNU-120596; Contra = contralateral; Ipsi = ipsilateral.

### 2.3.16 The Effects of i.t. Administration of the MEK Inhibitor U-0126 on PNU-120596's Antinociceptive Effect in the Formalin Test

We next investigated whether the antinociceptive effect of PNU-120596 was mediated through ERK activation in mice. Pretreatment of mice with i.t. injection of gradually increasing doses of U0126, a selective MEK inhibitor (Kominato et al., 2003; Aley et al., 2001) , on its own did not affect nociceptive responses induced by formalin injection in U0126-treated mice compared to their vehicle controls at any of the doses tested (0.04, 0.2 and 1  $\mu\text{g}/\text{mouse}$ , i.t.) (Figure 16). However, U0126 administered 5 min before PNU-120596 (4mg/kg, i.p.) dose-dependently blocked [ $F_{(7,36)} = 13.38$ ,  $p < 0.0001$ ] PNU-120596-induced antinociception in phase II of the formalin test. U0126 at the dose of 1  $\mu\text{g}/\text{mouse}$  completely reversed PNU-120596's actions in the formalin test.



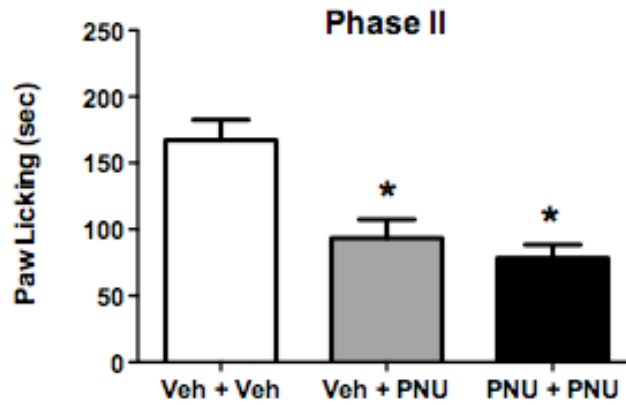
**Figure 16. Effects of MEK inhibitor, U0126, on PNU-120596-induced antinociception in the formalin test.**

Mice were pretreated with the MEK inhibitor U0126 (0.04, 0.2 and 1 $\mu$ g/mouse, i.t.) or vehicle 5 min prior to PNU-120596 (4mg/kg., i.p.) injection. Mice were tested 15 min later in the phase II of the formalin test. Each bar represents the mean  $\pm$  S.E.M for each group of 6-8 mice. \* $p < 0.05$  versus vehicle-vehicle group; #,  $p < 0.05$  versus 4mg/kg PNU-120596. Veh = vehicle; PNU = PNU-120596.

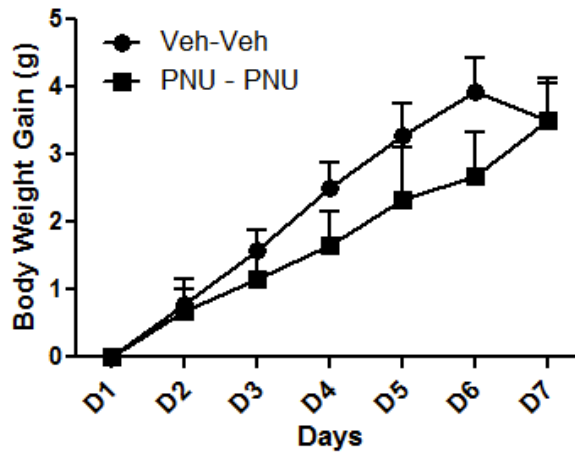
### 2.3.17 Tolerance did not Develop to PNU-120596's Effects in the Formalin Test after Chronic Exposure

We investigated if tolerance develops to PNU-120596's antinociceptive effects after chronic exposure of the drug. The dosing protocol of PNU-120596 was based on the time-course study of the drug shown earlier (24 hr long). Animals were treated with either vehicle or PNU-120596 (4 mg/kg, i.p.) once a day (8:00 am) for 6 days. On day 7, mice were challenged with PNU (4 mg/kg, i.p.) and tested 15 min later. As seen in Figure 17A, tolerance did not develop [ $F_{(2,15)} = 17.89$ ,  $p < 0.0001$ ] after chronic exposure to PNU-120596 in phase II of the formalin test. Furthermore, the chronic treatment of PNU-120596 did not significantly change the weight gain of mice compared to the vehicle-treated group (Figure 17B).

(A)



(B)



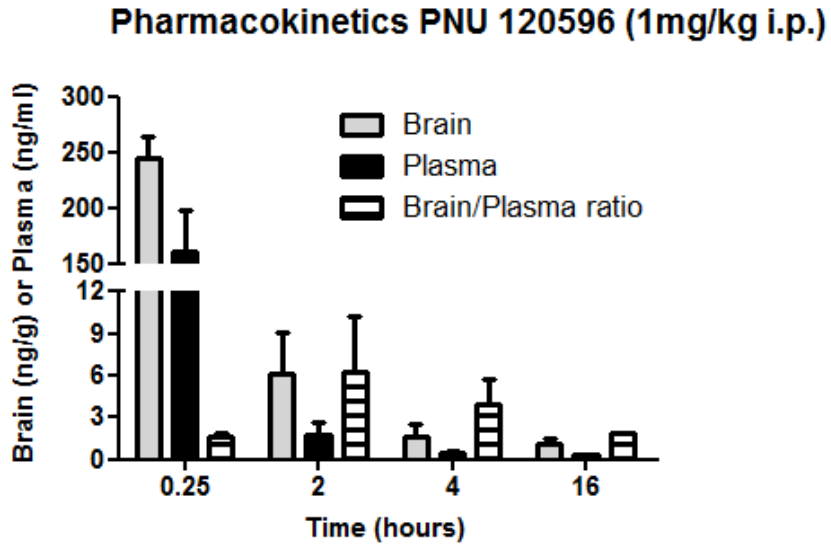
**Figure 17. Lack of tolerance to PNU-120596-induced antinociception in the formalin test after chronic administration of the drug.**

(A) Mice were treated with either vehicle or PNU-120596 (4 mg/kg, i.p.) once a day for 6 days. On day 7, mice were challenged with PNU (4 mg/kg, i.p.) and tested 15 min after in the phase II of the formalin test. Control group received vehicle for 6 days and were challenged on day 7 with vehicle and tested 15 min later in the formalin test. (B) Lack of significant effect on the body weight change of mice after chronic injection PNU-120596. Body weight change (Body weight at the injection day – Initial Body weight before treatment) in the two treatment groups was recorded daily at the same time. Each bar represents the mean  $\pm$  S.E.M for each group of 6-8 mice. \* $p < 0.05$  versus vehicle-vehicle group. Veh = vehicle; PNU = PNU-120596.

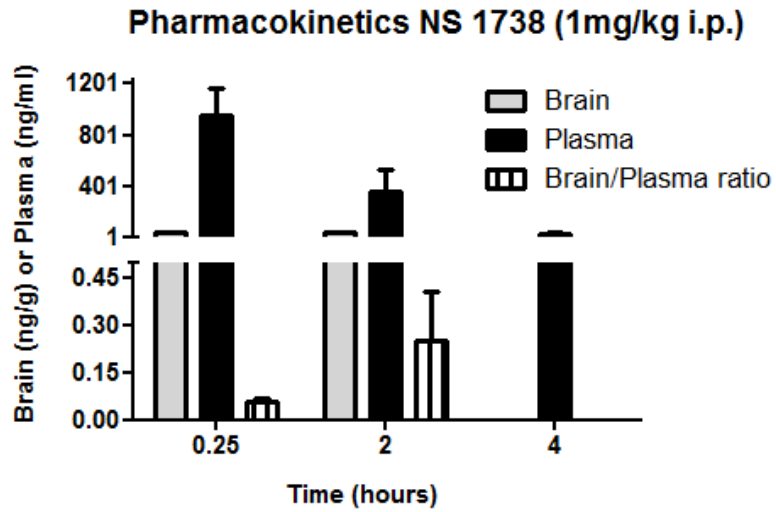
### 2.3.18 Measurement of $\alpha 7$ type I and II PAMs Levels in the Brain and Plasma

To estimate the ability of PAMs to permeate the blood-brain barrier, mice were administered 1 mg/kg of either NS 1738 or PNU-120596 i.p. Brain and plasma concentrations were measured at times 0.25, 2, 4 and 16 hr for PNU-120596 after dosing and it corresponded to 150 to 250 ng/ml in plasma versus brain, respectively at the 0.25 hr time point and less with the latter time points. By the time point of 4 hr after PNU-120596 injection, brain and plasma levels were close to 2 ng/ml (Figure 18A). Brain and plasma concentrations were measured at times 0.25, 2 and 4 hr for afer 1 mg/kg i.p. of NS 1738 which yielded only 1 ng/ml in brain versus 1000 ng/ml in plasma samples (Figure 18B). To assess if pharmacokinetic factors are responsible for the lack of pharmacological effects for NS-1738, a 30 mg/kg i.p. dose was also assessed and brain to plasma ratio estimated at 15 min time point. As shown in Figure 18C, sufficient NS1738 (close to 300 ng/ml) crosses the blood-brain ratio to further support pharmacodynamic differences between type I and II PAMs in regards to antinociceptive efficacy.

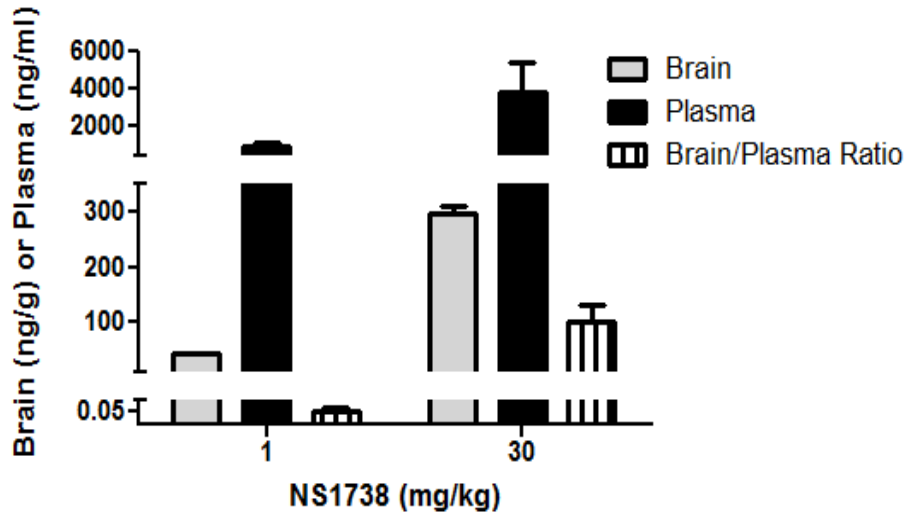
(A)



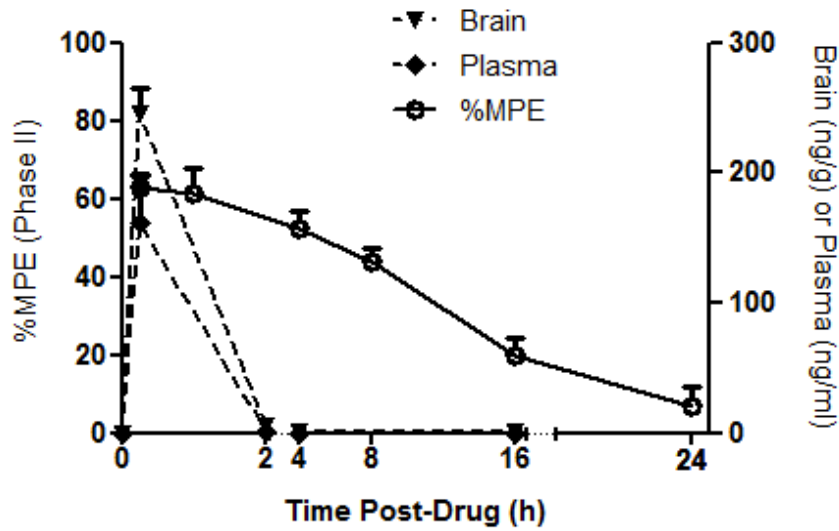
(B)



(C)



(D)





**Figure 18. Pharmacokinetics of NS-1738 and PNU-120596.**

(A) Mice (n=3) were dosed with 1 mg/kg i.p. of PNU-120596 at time 0, and both brain and plasma samples were collected at 0.25, 2, 4 and 16 hours and analyzed by mass spectrometry for PNU-120596 content in order to estimate the brain/plasma ratio at each time point. (B) Mice (n=3) were dosed with 1 mg/kg i.p. NS-1738 at time 0 and both brain and plasma samples were collected at times 0.25, 2 and 4 hours. (C) To parallel in vivo efficacious doses, mice (n=3) were dosed with either 1 mg/kg or 30 mg/kg i. p. NS-1738 at time 0 and both brain and plasma samples were collected at 15 min to compare levels. (D). Pharmacokinetic-Pharmacodynamic (PK-PD) relationships of PNU-120596. Time course of efficacy and relation to plasma and brain concentrations of PNU-120596. Antinociception [% Maximum Possible Effect (%MPE)] was assessed 0.25, 2, 4, 8, 16 and 24 h postdose of 4 mg/kg (left y-axis). Plasma and brain levels of PNU-120596 were determined in a satellite group of mice (plotted along the right y-axis). Although plasma and brain concentrations of PNU-120596 decreased to levels below 5 ng/ml by 2 h, significant effect in the formalin test still persists ( $p < 0.05$  versus vehicle controls). All the data are represented as mean  $\pm$  S.E.M.

## 2.4 Summary of Chapter II Results

In this study, we evaluated the antinociceptive activity of  $\alpha 7$  nAChRs PAMs NS1738 and PNU-120596 in animal models of acute and tonic pain behavior. Our studies show that similar to  $\alpha 7$  agonists,  $\alpha 7$  PAMs are not active in acute thermal pain tests (hot-plate and tail-flick) and mechanical sensitivity tests, after systemic administration in mice

In the formalin test, NS1738 marginally reduced tonic pain behavior in phase I, but not in phase II. However, PNU-120596 displayed significant and long-lasting effects in the both phases of the test, as showed by dose-response and time-course analyses. Similarly, the selective  $\alpha 7$  nAChR agonist PHA-543613, at a narrow range of doses, reduced nociceptive behavior in the formalin test. Additionally, PNU-120596, at antinociceptive doses, had no effect on locomotor activity and motor coordination in mice. Indeed, tolerance to PNU-120596's effects in the formalin test did not develop after chronic exposure

The  $\alpha 7$  antagonist MLA blocked effects of PNU-120596 in the formalin test, while  $\beta 2$ -nAChR-containing selective antagonist DH $\beta$ E did not. In addition, genetic deletion of  $\alpha 7$  nAChR reduced PNU-120596' antinociceptive effects. However, opioid receptor antagonist naloxone did not alter PNU-120596's effect in the formalin test. These results suggest that modulation of  $\alpha 7$  nAChRs by PNU-120596 was  $\alpha 7$  receptor-mediated.

Furthermore, central and peripheral site seems to mediate PNU-120596's effect in the formalin test. Indeed, PNU-120596 was found to have an effect after i.p. and i.t. administration in the formalin test, whereas NS1738 had no effect. Furthermore, MLA (i.t.) reversed PNU-120596 (i.p.) while MLA (i.pl.) partially blocked PNU-120596 (i.p.) in the ipsilateral paw of that received formalin (i.pl.), however MLA produced no effect on PNU-120596's actions in the

contralateral paw. Both PNU-120596 and NS1738 were found to cross the blood-brain barrier and diminish in concentration over time. The long-acting effect of PNU-120596 in the formalin test cannot be simply explained by the pharmacokinetic profile of the drug. Indeed as shown in Figure 18, most of the PNU-120596 is eliminated from plasma and brain of the animals 4 hr after injection of the drug. These findings indicate discordance between the Pharmacokinetic-Pharmacodynamic (Figure 18D) properties of PNU-120596 that may represent a key mechanistic feature of the antinociceptive efficacy associated with  $\alpha 7$  nAChRs positive allosteric modulation.

Our results additionally show that MEK inhibitor U0126 produced no effect in the formalin test, although dose-dependently blocked, and at our highest administered dose, completely reversed, PNU-120596's effects in phase II of the formalin test.

## CHAPTER III

### **In Vivo Pharmacological Interactions Between Type II Positive Allosteric Modulator of $\alpha_7$ Nicotinic Acetylcholine Receptors and Nicotinic Agonists in Mice**

#### **3.1 Chapter III Introduction**

Endogenous cholinergic neurotransmission, which is mediated by ACh and choline (Zhu et al., 2009), is a strong candidate for enhancement by  $\alpha_7$  nAChR PAMs. While  $\alpha_7$  PAMs were reported to enhance the actions of ACh in *in vitro*  $\alpha_7$  nAChRs expressed systems (Bertrand and Gopalakrishnan, 2007), the rapid clearance of ACh by acetylcholinesterases in the synaptic cleft makes choline, an ACh precursor and metabolite, a more likely candidate (Alkondon and Albuquerque, 2006). Indeed, choline is a selective endogenous agonist for  $\alpha_7$  nAChRs in the CNS (Alkondon et al., 1997; González-Rubio et al., 2006). Furthermore, PNU-120596 was recently reported to enhance choline's effects on native and expressed  $\alpha_7$  nAChRs (Kalappa et al., 2010; Gusev and Uteshev 2010). In addition, exogenous choline administered centrally has been known to produce antinociception mediated by  $\alpha_7$  nAChR in various animal models of pain (Damaj et al., 2000; Wang et al., 2005). Therefore, in this study, we used choline to investigate the effects of PNU-120596 on an endogenous  $\alpha_7$  agonist in a mouse model of pain. Choline is a charged cation and cannot easily pass through the blood–brain barrier (BBB) (Allen and Lockman, 2003), so the interaction with  $\alpha_7$  PAMs was evaluated by intrathecally administered choline.

The present study investigated the effects of PNU-120596 administered alone or in combination with nicotinic agonists in the mouse formalin test, a model of persistent pain (Hunskar and Hole, 1987). Studies were designed to test the working hypotheses that (a) PNU-

120596 administered alone would produce antinociception, and (b) PNU-120596 would synergistically enhance the effects of the  $\alpha 7$  nAChR agonist choline. In particular, we assessed the nature of the interaction between PNU-120596 and choline in the formalin test by performing an isobolographic analysis (Tallarida, 2001; Tallarida, 2006; Gessner., 1995; Tallarida and Raffa, 2010; Ossipov et al., 1990). In addition, we evaluated the interaction of PNU-120596 with PHA-54613, an exogenous selective  $\alpha 7$  nAChR agonist, in the formalin test. Finally, considering that PNU-120596's potentiation of nicotine's adverse effects might be a clinical concern for smokers and patients undergoing  $\alpha 7$  nAChR agonist-based nicotine-replacement therapy (Brunzell and McIntosh, 2012), we assessed the interaction between PNU-120596 and nicotine using acute thermal pain tests (tail-flick and hot-plate tests), locomotor activity, body temperature and convulsing activity in mice.

## 3.2 Materials and Methods

### 3.2.1 Animals

Male adult ICR mice obtained from Harlan Laboratories (Indianapolis, IN) were used throughout the study. All other animal parameters including housing, care and demographics were the same as described in chapter II.

### 3.2.2 Drugs

(-)-Nicotine hydrogen tartrate salt [(-)-1-methyl-2-(3-pyridyl) pyrrolidine (-)-bitartrate salt] was purchased from Sigma-Aldrich (St. Louis, MO). MLA and choline were purchased from RBI (Natick, MA). PNU-120596 [1-(5-Chloro-2,4-dimethoxy-phenyl)-3-(5-methyl-isoxazol-3-yl), PHA-543613 (selective  $\alpha 7$  agonist) and morphine were obtained from the Drug Supply Program of the National Institute on Drug Abuse (Rockville, MD). Both, choline and

morphine were dissolved in physiological saline (0.9% sodium chloride) and injected intrathecally or subcutaneously respectively, at a total volume of 1ml/100 g body weight unless noted otherwise.

### 3.2.3 Antinociceptive Tests

#### 3.2.3.1 *Drug Interactions in the Formalin Test*

The Formalin test was conducted as described in chapter II.

To evaluate the nature of interaction between PNU-120596 and choline, dose-response curves for PNU-120596 and choline were obtained using at least six animals at each dose. Mice were given i.p. injections of either vehicle or PNU-120596 (0.1, 0.32, 1.0, 3.2 or 10 mg/kg), or i.t injections of vehicle or choline (1, 3.2, 10, 32 or 100 mg/kg) and 20 min later were given an i.pl. injection of formalin. Dose response curves were also obtained after coadministration of PNU-120596 and choline ( $ED_{50 \text{ Mix}}$ ) in fixed-ratio combinations based on fractions of their respective  $ED_{50}$  values. The ratio of PNU-120596 and choline was 1:7.2 (see Table 1). In the drug combination experiments, mice received one injection of each drug: PNU-120596 (i.p.) was administered first, followed 15 min later by a choline (i.t.) injection. Formalin (i.pl.) was administered 5 min after the choline injection. For the  $ED_{50}$  values estimation, data were expressed as % MPE = (licking time, vehicle – licking time, test drug)/(licking time, vehicle) x 100.

For the interactions studies of PNU-120596 with other agonists, PNU-120596 was administered via i.p., whereas nicotine, PHA-543613 and morphine were given via s.c. injection. Nicotine was given 5 min after PNU-120596 treatment. PHA-543613 and morphine were given 15 min after PNU-120596 treatment.

### 3.2.3.2 Tail-Flick Test

The antinociceptive effect of drugs was assessed by the tail-flick method, as described in chapter II. Groups of 6 animals were used for each dose and for each treatment. Interaction studies were carried out by pretreating the mice with either vehicle or PNU-120596 15 min before vehicle or nicotine (0.5 and 2.5 mg/kg s.c.) and animals were tested 5 min later.

### 3.2.3.3 Hot-Plate Test

The hot-plate test was utilized as described in chapter II. Interaction studies were carried out by pretreating the mice with either vehicle or PNU-120596 15 minutes before vehicle or nicotine (0.5 and 2.5 mg/kg s.c.) 5 minute pretreatment.

### 3.2.4 Body Temperature Measure

Rectal temperature was measured by a thermistor probe (inserted 24 mm) and digital thermometer (YSI Inc., Yellow Springs, OH). In this set of experiments readings were taken just before and 30 min after the last injection. In the first experiment, mice were pretreated with an s.c injection of either vehicle or nicotine (0.5, 1 or 2.5mg/kg) and were tested 30 min later. PNU-120596 was given 15 min before nicotine treatment. In the second experiment mice were pretreated with either vehicle or different doses of PNU (1, 4 or 8 mg/kg, i.p.), and 15 min later they received s.c injection of veh or nicotine 0.5 mg/kg. Mice were tested 30 min after the second injection. In the third experiment mice were pretreated 15 min with either vehicle or PNU-120596 and tested 15 min after a second injection of either vehicle or PHA-543613. Antagonism studies were carried out by pretreating the mice with either vehicle or MLA (10mg/kg) 10 min before vehicle or PNU-120596 (8mg/kg). Followed by a 15 min pretreatment of vehicle or PNU-120596, the animals were tested 30 min after administration of a

subcutaneous dose of 0.5 mg/kg of nicotine. The difference in rectal temperature before and after treatment was calculated for each mouse. The ambient temperature of the laboratory varied from 21 to 24°C from day to day.

### 3.2.5 Locomotor Activity Test

Mice were placed into individual Omnitech photocell activity cages (28 x 16.5 cm) after the pretreatment of either i.p. vehicle or PNU-120596 (8mg/kg) and an s.c. injection of either vehicle or nicotine (0.1, 0.5, and 1mg/kg). The initial pretreatment, of PNU-120596 or vehicle, was given 15 minutes before the second pretreatment, of either vehicle or nicotine, which was administered 5 minutes before the test. Interruptions of the photocell beams (two banks of eight cells each) were then recorded for the next 30 min. Data are expressed as number of photocell interruptions.

### 3.2.6 Seizure Testing

After an s.c. pretreatment injection of either vehicle or nicotine (3, 5 and 9 mg/kg), each animal was immediately placed in a 30x30x30 cm<sup>3</sup> Plexiglas cage for 3 min, after which mice were observed for 7 min. The interaction study between PNU-120596 and nicotine was carried out by pretreating the mice i.p with PNU-120596 15 min before nicotine. The occurrence or absence of a clonic seizure was noted for each animal over a 7-min time period after the s.c. administration of nicotine. This time was chosen because seizures occur very quickly after nicotine administration. The percentage of animals exhibiting a seizure was calculated.

### 3.2.7 Intrathecal Injections

Injections were administered as described in chapter II.



### 3.2.8 Statistical Analysis

Results are expressed as mean  $\pm$  standard error of the mean (SEM) or as ED<sub>50</sub> values with 95% confidence limits. The data obtained were analyzed using GraphPad<sup>®</sup> software program. Statistical analysis was done by one-way analysis of variance test ANOVA followed by post hoc, Tukey test. *P*-values less than 0.05 ( $P < 0.05$ ) were considered significant. The ED<sub>50</sub> values with 95% confidence intervals were calculated using standard linear regression analysis of the dose-response curve for each drug alone or in combination. To determine the ED<sub>50</sub> values, the antinociceptive activity (reduction in paw licking) was calculated as a %MPE using the following equation: %MPE = 100\*((mean paw licking in control group)–(mean paw licking in drug(s) treated group))/mean of paw licking in control group).

Isobolographic analysis was used to determine the nature of the drug interactions, as described previously (Tallarida, 2000). The dose of PNU-120596 required to elicit a 50% effect was plotted on the abscissa, and the isoeffective dose of choline was plotted on the ordinate. The theoretical additive effect of the two drugs was represented by the straight line connecting the two points. If the experimentally determined data points and their confidence interval lie on this line, the drug effects are considered additive. If the points lie below this line, the interaction is considered to be superadditive (synergistic); however, if they lie above the line of additivity, the interaction is defined as subadditive (antagonistic). To determine whether the interaction between two drugs was synergistic, additive, or antagonistic, the theoretical additive ED<sub>50</sub> value of the two drugs combined (referred to as  $Z_{add}$ ) was calculated from the dose-response curves of each drug administered individually, in which the combination is assumed to equal the sum of the individual effects of each drug. The experimental ED<sub>50</sub> value of the two drugs in combination (referred to as  $Z_{mix}$ ) in which the two drugs were summed at each concentration was then

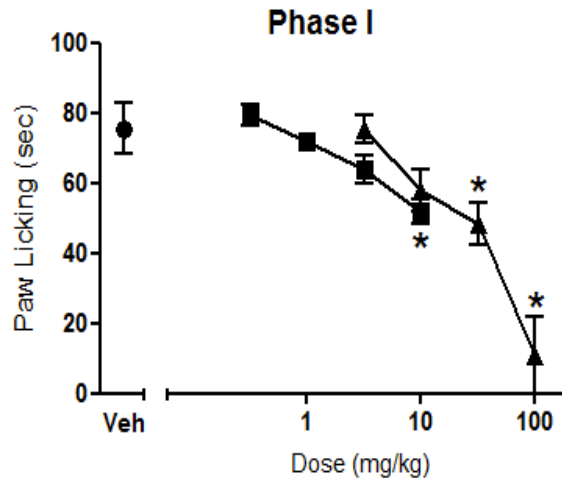
determined by linear regression. If the experimentally determined  $Z_{mix}$  value is significantly ( $p < 0.05$ ) lower than the predicted  $Z_{add}$  value, the drugs were said to act synergistically.

### 3.3 Results

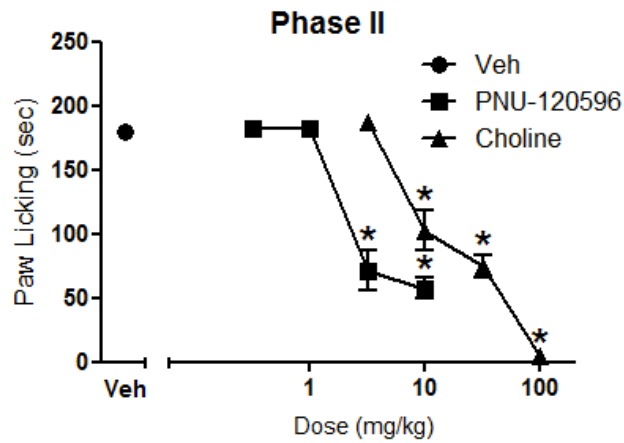
#### 3.3.1 Dose-response Analysis of PNU-120596 and Choline Alone and Their Combinations in the Formalin Test

Intraplantar injection of formalin into the mouse's hind paw resulted in a reliable biphasic licking response induced by a tonic biphasic inflammatory response. PNU-120596 (i.p) and choline (i.t.) dose-dependently reduced formalin nociceptive behaviors in both phase I and II. Figure 19 (A & B) shows the dose-response curves for the antinociceptive effects of PNU-120596 in phase I [ $F_{(5,49)} = 14.57, p < 0.0001$ ] and phase II [ $F_{(5,49)} = 54.43, p < 0.0001$ ] and choline phase I [ $F_{(5,49)} = 21.16, p < 0.0001$ ] and phase II [ $F_{(5,49)} = 64.91, p < 0.0001$ ] alone in mice. The  $ED_{50}$  values and 95% confidence limit for PNU-120596 and choline in phase II were 2.61 (2.13-3.19) and 18.90 (14.79-24.17) mg/kg, respectively as shown in Table 1.

(A)



(B)



**Figure 19. Effects of  $\alpha 7$  type II PAM PNU-120596 and  $\alpha 7$  nicotinic agonist choline alone in the mouse formalin test of persistent pain.**

Dose-response curves of PNU-120596 and choline on (A) phase I and (B) phase II in the formalin test. Mice were treated with either vehicle or PNU-120596 (15 min, i.p.) or vehicle or choline (5 min, i.t.). Testing occurred immediately after the formalin injection. Each point represents the mean  $\pm$  S.E.M of total time spent licking for 6-8 mice per group and \*denotes  $p < 0.05$  vs. vehicle. Veh = vehicle.

Table 1

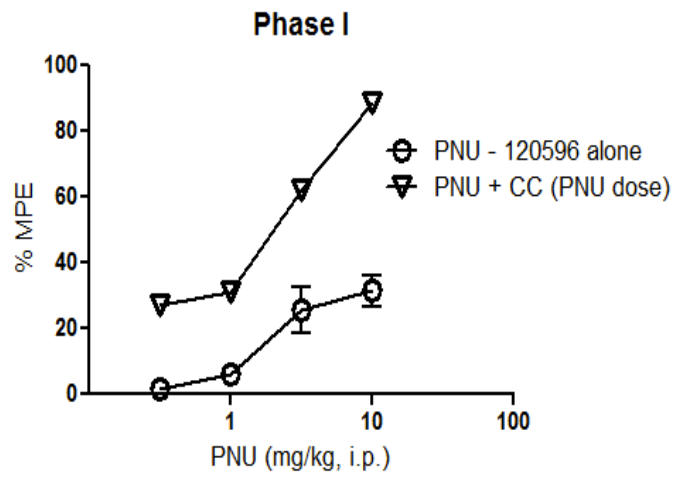
Treatment (Alone or Mixture) [PNU: mg/kg   Choline: µg/5µL]	Mixture Ratio	Theoretical ED <sub>50</sub> [Z <sub>add</sub> (95% CL)]	Experimental ED <sub>50</sub> [Z <sub>mix</sub> (95% CL)]
PNU Alone	----	----	2.61 (2.13-3.19)
Choline Alone	----	----	18.90 (14.79-24.17)
PNU-120596 (in PNU-120596 + Choline Mixture)	----	----	0.47 (0.28-0.78)
0.32			
1.0			
3.2			
10			
Choline (in PNU-120596 + Choline Mixture)	----	----	3.44 (2.36-4.99)
2.32			
7.2			
23.2			
72			
PNU-120596 + Choline	1:7.2	10.76 (8.71-13.18)	3.92 (2.70-5.68)
0.32 + 2.32			
1.0 + 7.2			
3.2 + 23.2			
10 + 72			

**Table 1.** Fixed combinations of PNU-120596 and choline produced synergistic antinociceptive effects in the formalin test. Predicted additive ED<sub>50</sub> values (Z<sub>add</sub>) and experimentally determined ED<sub>50</sub> (Z<sub>mix</sub>) values for the interaction of PNU-120596 and choline. Doses for each drug or drug interaction are presented in the first column.

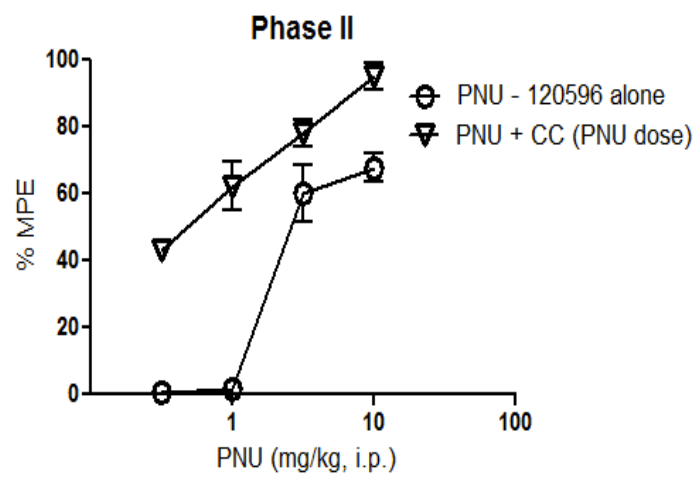
### 3.3.2 Dose-response Analysis of PNU-120596 and Choline Alone and Their Combinations in the Formalin Test

Shown in Figure 20 (A & B) is the 1:7.2 combination of PNU-120596 and choline, with the dose of PNU-120596 plotted on the abscissa. The dose-response curve of choline alone is plotted in this graph for comparison. The same data are also plotted in Figure 20 (C & D), with the dose of choline plotted on the abscissa. The dose-response curve of PNU-120596 alone is included in this graph for comparison. The combination of PNU-120596 with choline resulted in leftward shift of the dose-response curve with regard to choline only. The interaction between PNU-120596 (i.p., 15 min before the formalin injection) and choline (i.t., 5 min before the formalin injection) was assessed by an isobolographic analysis. The plots of the experimental combination  $ED_{50}$  values for both fixed ratios in relation to the  $ED_{50}$  values of the drugs alone are shown in Figure 21. The isobologram graphic suggests that a synergistic interaction occurs between PNU-120596 and choline since the experimental  $ED_{50}$  is located below the additive dose line. The synergistic interaction between PNU-120596 and choline is confirmed by statistical analysis of the predicted additive  $ED_{50}$  values ( $Z_{add}$ ) and experimentally derived  $ED_{50}$  values ( $Z_{mix}$ ) as shown in Table I. The ratio tested showed that  $Z_{mix}$  value is significantly less than the  $Z_{add}$  value. These data indicate that the interaction between the antinociceptive actions of PNU-120596 and choline synergistic in the formalin test.

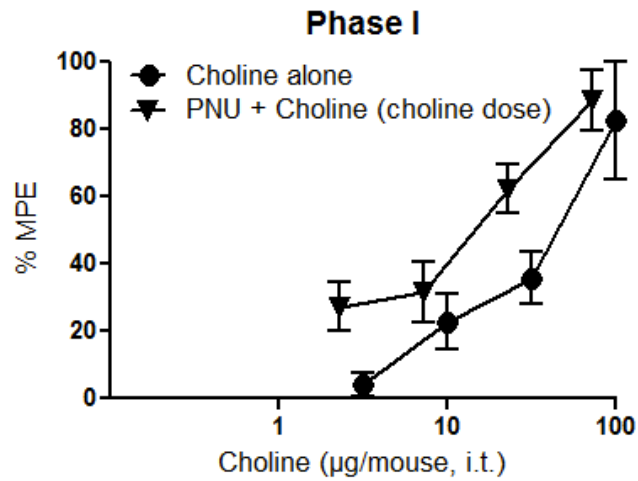
(A)



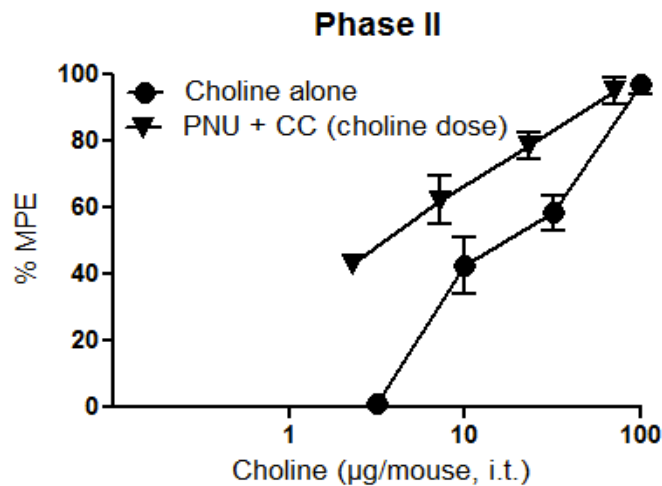
(B)



(C)

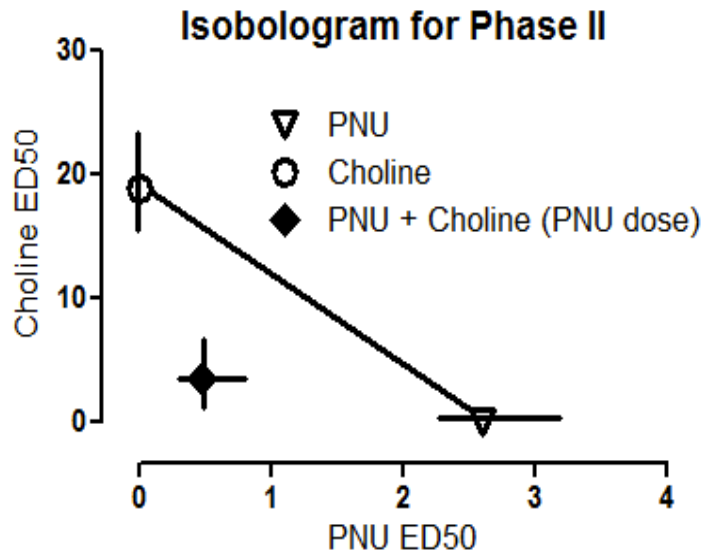


(D)



**Figure 20. Effects of the interaction of PNU-120596 with choline in the formalin test, with respect to each drugs dose.**

Dose-response curves for PNU alone and in combination with choline in (A) phase I and (B) phase II in the formalin test, plotted against the doses of PNU-120596. Mice treated with either vehicle or PNU-120596 15 min before vehicle or choline, which was given 5 min before formalin (i.pl.). Dose-response curves for choline alone and in combination with PNU-120596 in (C) phase I and (D) phase II in the formalin test, plotted against choline doses. Testing occurred immediately after the formalin injection. Each point represents the mean  $\pm$  S.E.M of total time spent licking for 6-8 mice per group and \*denotes  $p < 0.05$  vs. vehicle. Veh = vehicle.



**Figure 21. ED<sub>50</sub> isobologram for the interaction of PNU-120596 and choline in the formalin test.**

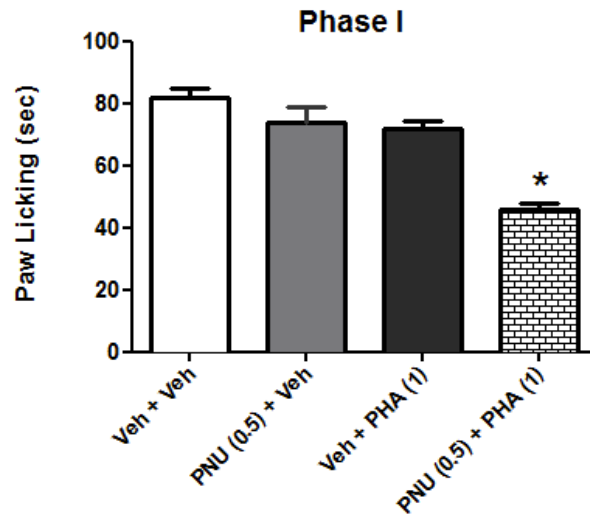
The ED<sub>50</sub> values for PNU-120596 and choline are depicted on the x- and y-axes, respectively. The experimental ED<sub>50</sub> values of mixtures of PNU-120596 and choline at fixed-ratio combinations of 1:7.2, with 95% confidence intervals, were significantly below the theoretical isobole of additivity (see Table 1), indicating a synergistic interaction. Each point represents the mean ± SEM for 6 animals. \*p < 0.05, compared with control mice given vehicle. Z<sub>mix</sub> corresponds to experimental ED<sub>50</sub> ± SEM of the combination with 95% confidence limits.



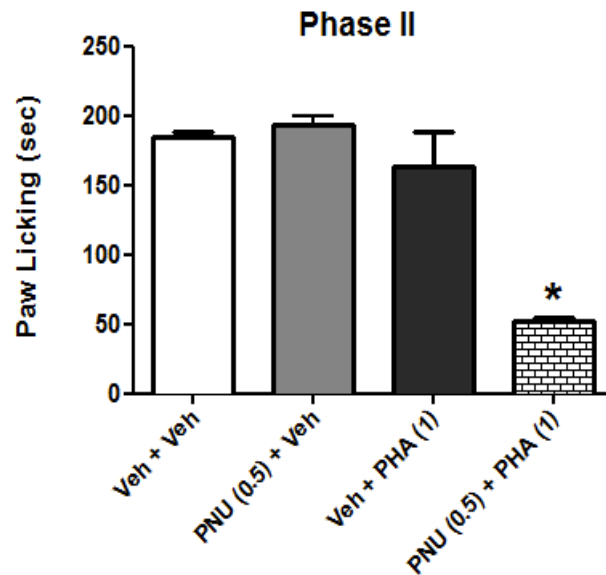
### 3.3.3 PNU-120596 Enhances PHA-543613's Effects in the Formalin Test

Since PNU-120596 was shown to enhance the antinociceptive effect of choline, an endogenous  $\alpha 7$  nAChR agonist, we evaluated if it also enhances the effect of an exogenous selective  $\alpha 7$  nAChR agonist, such as PHA-54613. Therefore, we tested PNU-120596 and PHA-54613 for their effects, separately and in combination, in the formalin test. Low doses of PNU-120596 (0.5 mg/kg i.p.) and PHA-543613 (1.0mg/kg i.p.), both administered alone and given 15 min before formalin, did not produce significant antinociceptive effects in phases I [ $F_{(2,18)} = 2.449$ ,  $p = 0.1146$ ] and II [ $F_{(2,17)} = 0.4704$ ,  $p = 0.6327$ ] of the formalin test (Figure 22A & B). On the other hand, pretreatment with PNU-120596 significantly potentiated PHA-543613's effects in both phase I [ $F_{(3,22)} = 23.72$ ,  $p < 0.0001$ ] and phase II [ $F_{(3,22)} = 9.468$ ,  $p = 0.0003$ ] of the formalin test (Figure 22A & B).

(A)



(B)

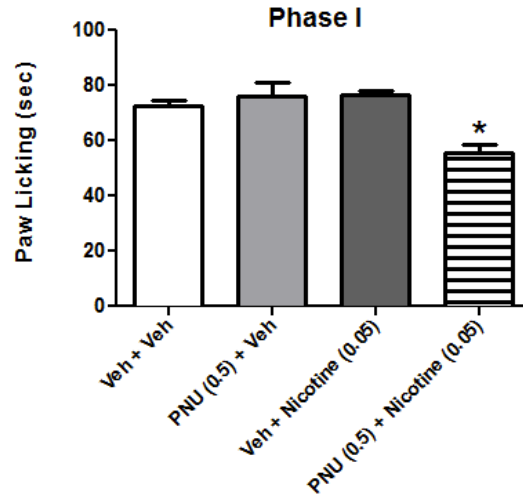


**Figure 22. PNU-120596 enhances PHA-543613's effects in the formalin test.** Mice received vehicle or PNU-120596 ( $0.5 \text{ mg kg}^{-1}$ , i.p.) and 15 min later PNU-120596 ( $0.5 \text{ mg kg}^{-1}$ , s.c.). They were tested in the formalin test 15 min later. Paw-licking time was measured in both (A) phase I and (B) phase II of the test. Data are expressed as means  $\pm$  SEM;  $n = 6$  mice per group. \* $p < 0.05$  vs. control group.

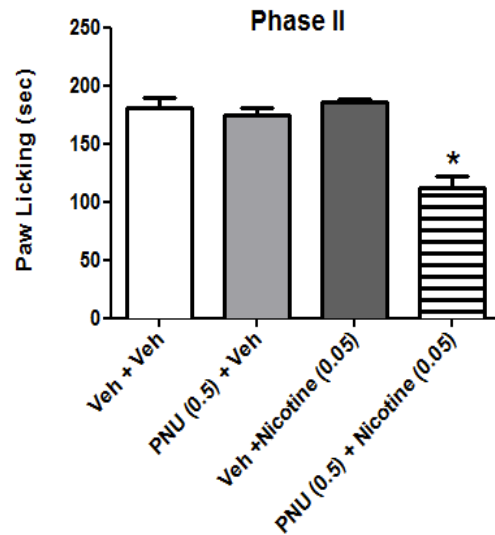
### 3.3.4 PNU-120596 Enhances Nicotine's Effects in the Formalin Test

To further explore the interaction between PNU-120596 and nicotinic agents, PNU-120596's effects on nicotine's antinociception in the formalin test were also studied. As shown in Figure 23 (A & B), neither PNU-120596 (0.5mg/kg i.p.), administered 15 min before formalin, nor nicotine (0.05mg/kg s.c.), administered 5 min before formalin, induced significant antinociceptive effects in phase I [ $F_{(3,21)} = 0.610$ ,  $p = 0.3218$ ] or II [ $F_{(3,21)} = 1.029$ ,  $p = 0.3746$ ] on their own in the formalin test. However, pretreatment of mice with PNU-120596 significantly enhanced nicotine's effects in the phase I [ $F_{(3,28)} = 13.59$ ,  $p < 0.0001$ ] and II [ $F_{(3,25)} = 34.64$ ,  $p < 0.0001$ ] of the formalin test (Figure 23A & B).

(A)



(B)

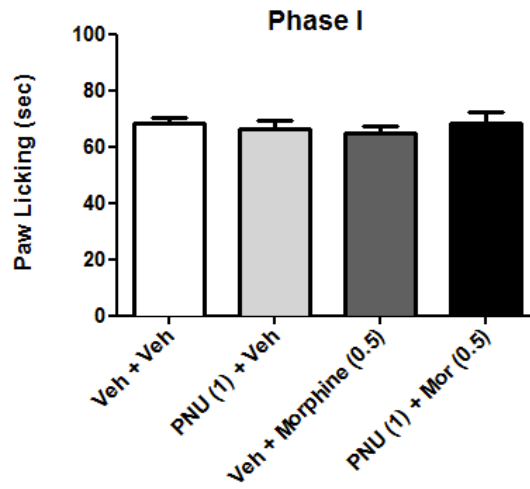


**Figure 23. PNU-120596 enhances nicotine's effects in the formalin test.** Mice received vehicle or PNU-120596 ( $0.5 \text{ mg kg}^{-1}$ , i.p.) and 15 min later nicotine ( $0.05 \text{ mg kg}^{-1}$ , s.c.). They were tested in the formalin test 5 min later. Paw-licking time was measured in both (A) phase I and (B) phase II of the test. Data are expressed as means  $\pm$  SEM;  $n = 6$  mice per group. \* $p < 0.05$  vs. control group.

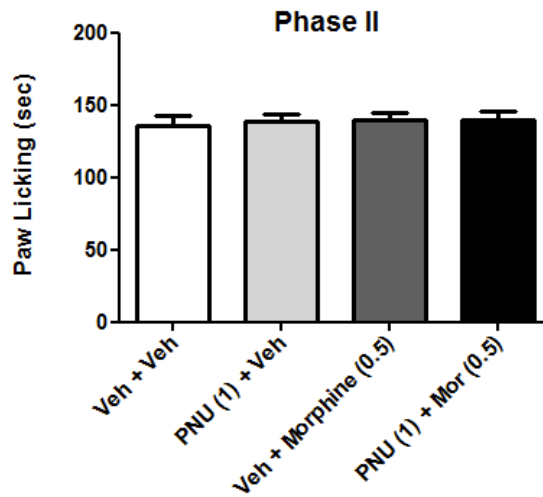
### 3.3.5 PNU-120596 Failed to Enhance Morphine's Effects in the Formalin Test

Considering the implication of  $\mu$ -opioid receptors in nociception, we investigated the interaction between PNU-120596 and morphine in the formalin test. Neither a single pretreatment (15 min) injection of PNU-120596 (1mg/kg, i.p.) nor a pretreatment (10 min) injection of morphine (0.5mg/kg, s.c.) produced a significant antinociceptive effect in formalin phases I [ $F_{(2,13)} = 0.5056$ ,  $p = 0.6145$ ] and II [ $F_{(2,13)} = 0.5056$ ,  $p = 0.6145$ ], compared to the vehicle group. To then assess the effect of PNU-120596 on morphine, mice were injected with PNU-120596 15 min (1mg/kg, i.p.) before they were injected with morphine (0.5 mg/kg, s.c.), which was given 15 min before the formalin injection (i.pl.). As shown in Figure 24 (A & B), interaction of ineffective doses of PNU-120596 and morphine did not produce antinociceptive effects in either phase I [ $F_{(3,19)} = 0.3993$ ,  $p = 0.7551$ ] or phase II [ $F_{(3,19)} = 0.09381$ ,  $p = 0.9625$ ] of the formalin test.

(A)



(B)



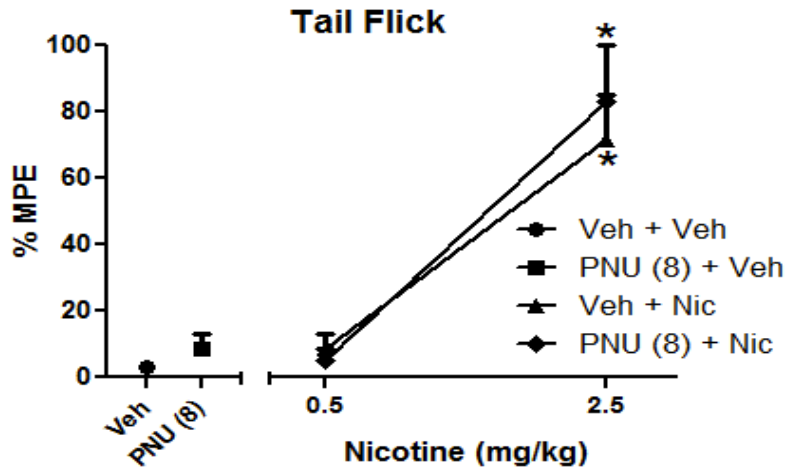
**Figure 24. PNU-120596 did not enhance morphine's effects in the formalin test.** Mice received vehicle or PNU-120596 ( $0.5 \text{ mg kg}^{-1}$ , i.p.) and 15 min later morphine ( $0.5 \text{ mg kg}^{-1}$ , s.c.). They were tested in the formalin test 15 min later. Paw-licking time was measured in both (A) phase I and (B) phase II of the test. Data are expressed as means  $\pm$  SEM;  $n = 6$  mice per group. \* $p < 0.05$  vs. control group.

### 3.3.6 The Effects of PNU-120596 on Nicotine-induced Antinociception

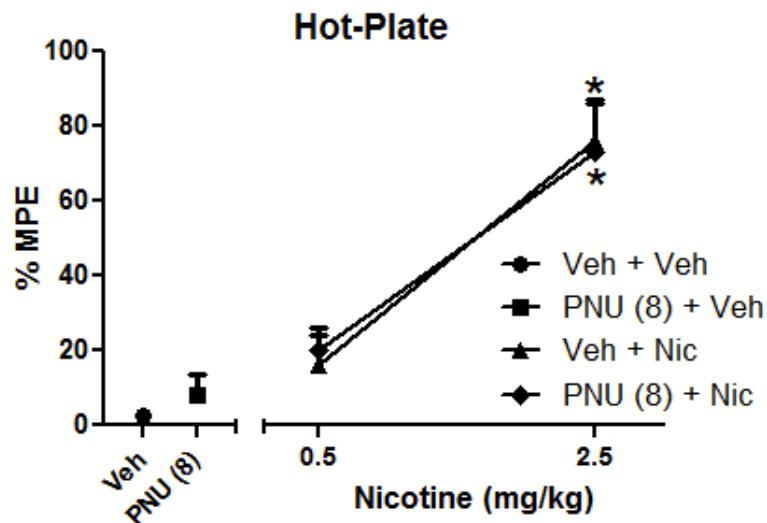
To further assess the interaction between PNU-120596 and nicotine, we determined the effects of PNU-120596 on various pharmacological responses after acute injection of nicotine in mice.

We first assessed the effects of PNU-120596 on nicotine-induced antinociception in acute thermal pain models using the tail-flick and hot-plate tests. A 15 min pretreatment of PNU-120596 (8 mg/kg) or a 5 min pretreatment of nicotine (0.5 mg/kg) showed no significant antinociceptive effects in the [ $F_{(3,22)} = 0.5612, p = 0.6463$ ] tail-flick and [ $F_{(3,20)} = 2.000, p = 0.1465$ ] hot-plate tests when compared to vehicle. In contrast, a 5 minute pretreatment of a higher dose of nicotine (2.5 mg/kg) induced significant antinociceptive effects in the tail-flick [ $F_{(5,32)} = 16.91, p < 0.0001$ ] and hot-plate tests [ $F_{(5,30)} = 15.83, p < 0.0001$ ] when compared to vehicle (Figure 25 A&B). To determine whether PNU-120596 enhanced or blocked the antinociceptive effect of nicotine (0.5mg/kg s.c. and 2.5mg/kg s.c), mice were first pretreated (15 min) with PNU-120596 (8mg/kg i.p.) and then pretreated (5min) with nicotine (0.5mg/kg s.c. or 2.5mg/kg s.c). Results shown in Figure 25 (A & B) demonstrate that the pretreatment with PNU-120596 failed to significantly alter nicotine's responses in the tail-flick and hot-plate test.

(A)



(B)



**Figure 25. PNU-120596 did not enhance nicotine's antinociception in tail flick and hot-plate tests after acute administration.** Effects of PNU-120596 on nicotine in the (A) tail-flick and (B) hot-plate tests. Mice received vehicle or PNU-120596 ( $8\text{mg kg}^{-1}$ , i.p.) and 15 min later nicotine ( $0.5$  or  $2.5\text{ mg kg}^{-1}$ , s.c.). They were tested in the tail-flick and hot-plate tests 5 min later. Each point represents the mean  $\pm$  S.E.M of total time spent licking for 6 mice per group and \* denotes  $p < 0.05$  vs. vehicle. Veh = vehicle.

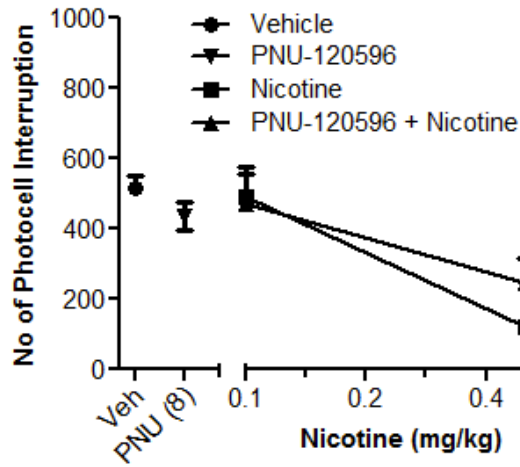


### 3.3.7 The Effects of PNU-120596 on Nicotine-decrease in Locomotion, Seizures and Hypothermia

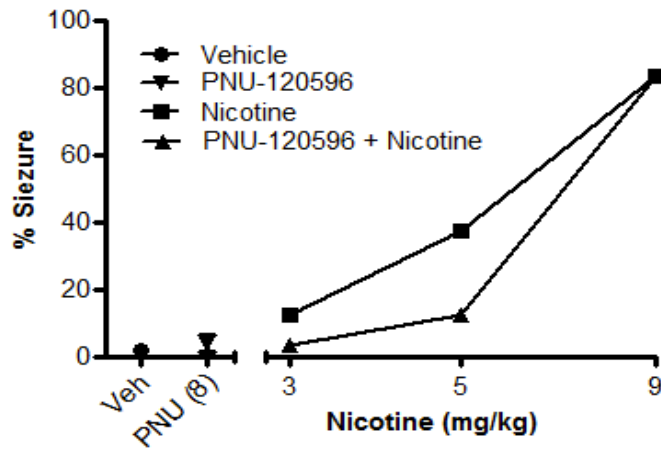
We then determined the effects of PNU-120596 on nicotine-induced decrease in locomotor activity in mice. Consistent with previous reports, nicotine produced a significant, dose-dependent reduction in motor activity (Berrendero et al., 2005; Damaj, 2001) (Figure 26A). A pretreatment of PNU-120596 (8 mg/kg) did not induce a significant shift in the nicotine dose-response curve compared to vehicle-treated group [ED<sub>50</sub> (±CL) values are 0.23 (0.07-0.73) and 0.33 (0.1-0.94) mg/kg for vehicle and PNU-120596-treated groups, respectively] (Table II). A similar lack of significant effects of PNU-120596 on nicotine-induced seizures (Figure 26B) was also observed [ED<sub>50</sub> (±CL) values are 6.4 (4.3-9.5) and 7.1 (6.4-7.7) mg/kg for vehicle and PNU-120596-treated groups, respectively] (Table II).

We finally investigated the effects of PNU-120596 on nicotine-induced hypothermia. Consistent with previous reports (Damaj et al., 1996), acute nicotine produced a significant and dose-dependent reduction in body temperature in mice (Figure 26C). While pretreatment of PNU-120596 (8 mg/kg) did not produce a significant leftward shift in the dose-response curve for nicotine-induced hypothermia [ED<sub>50</sub> (±CL) values are 0.47 (0.28-0.78) and 0.18 (0.09-0.35) mg/kg for vehicle and PNU-120596-treated groups, respectively] (Table II), significant interaction was found with the low dose of nicotine. As shown in Figure 26C, PNU-120596 (8 mg/kg i.p.), administered 15 min before 0.5 mg/kg of nicotine, induced a significant hypothermia. The enhancement of nicotine's effects disappeared at the higher doses of nicotine. On its own PNU-120596 (8 mg/kg i.p.) did not induce a significant change in the body temperature of mice compared to vehicle (Figure 26C).

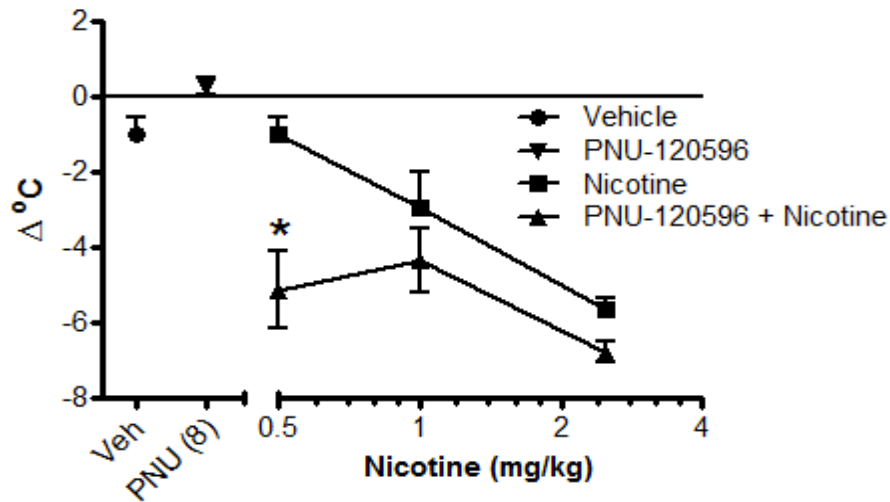
(A)



(B)



(C)



**Figure 26. Effects of PNU-120596 on nicotine-induced locomotor activity depression, seizure occurrence and hypothermia.** The effects of PNU-120596 ( $8\text{mg kg}^{-1}$ , i.p.) on nicotine's actions on (A) locomotor activity, (B) seizure occurrence and (C) body temperature measure in mice. Animals were treated with vehicle or PNU-120596 15 min before nicotine. Changes in locomotor activity and seizure occurrence were measured 5 min later. Changes in body temperature were measured 30 min later. Each point represents the mean  $\pm$  SEM of 6 mice. The asterisks denote the significance levels when compared with the control group: \* $p < 0.05$ .

Table 2

<b>ED50 (95% confidence limits) (mg/kg)</b>		
<b>Response</b>	<b>Vehicle</b>	<b>PNU-120596</b>
Spontaneous Activity	0.23 (0.07 – 0.73)	0.33 (0.1 – 0.94)
Seizures	6.4 (4.3 – 9.5)	7.1 (6.4 – 7.7)
Hypothermia	0.47 (0.28 – 0.78)	0.18 (0.09 – 0.35)

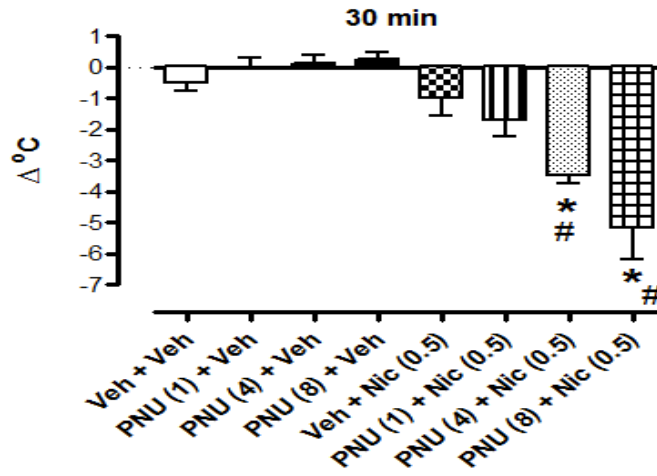
**Table. 2.** Summary of the potencies of various acute nicotine responses after pretreatment with PNU-120596. Potency is expressed as ED<sub>50</sub> ± confidence limits (mg/kg). Each group contained 6 mice.

### 3.3.8 Characterization of nicotine-induced hypothermia in the presence of PNU-120596

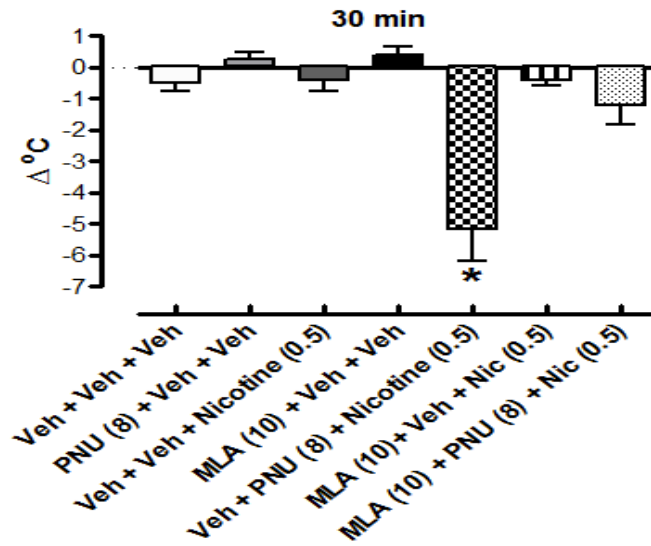
Having found the effect of PNU-120596-pretreated nicotine to be significantly different from the effect of just nicotine at a dose of 0.5 mg/kg, but not at 1.0 and 2.5 mg/kg, in the body temperature test, we chose this dose to further evaluate the interaction between PNU-120596 and nicotine. Mice were pretreated with various doses of PNU-120596 (1, 4 and 8 mg/kg i.p.) and 15 min later, injected with nicotine (0.5 mg/kg s.c.). Thirty min later changes in their body temperatures were measured. On their own, PNU-120596-, nicotine-, or vehicle-pretreated mice did not induce significant changes in body temperature [ $F_{(4,21)} = 2.059$ ,  $p = 0.1226$ ]. In contrast, PNU-120596 dose-dependently enhanced nicotine-induced hypothermia [ $F_{(7,37)} = 17.57$ ,  $p < 0.0001$ ] (Figure 27A). The enhancement of PNU-120596 of nicotine's hypothermic effects was mediated by  $\alpha 7$  nAChRs. Indeed, MLA, an  $\alpha 7$  nicotinic antagonist, blocked the effects of PNU-120596 on nicotine. As shown in Figure 27B, MLA (10 mg/kg, s.c.) given 5 min before, blocked the enhancement of nicotine-induced hypothermia (0.5 mg/kg s.c.) by PNU-120596 (8 mg/kg i.p.) [ $F_{(6,32)} = 14.34$ ,  $p < 0.0001$ ]. On their own, PNU-120596-, nicotine-, vehicle-, or MLA-pretreated mice did not induce significant changes in body temperature [ $F_{(3,18)} = 2.828$ ,  $p = 0.0677$ ].

Interestingly and in contrast to  $\alpha 7$  PAM PNU-120596, the  $\alpha 7$  nAChR agonist PHA-543613 (8 mg/kg s.c.) failed to enhance the effects of nicotine (0.5 mg/kg s.c.) on body temperature [ $F_{(3,18)} = 2.317$ ,  $p = 0.1100$ ] (Figure 27C). However, the combination of PNU-120596 and PHA-543613, produced significantly different hypothermic effects [ $F_{(5,29)} = 5.054$ ,  $p = 0.0019$ ], suggesting that PNU-120596 enhances the effect of PHA-543613 on body temperature (Figure 27C).

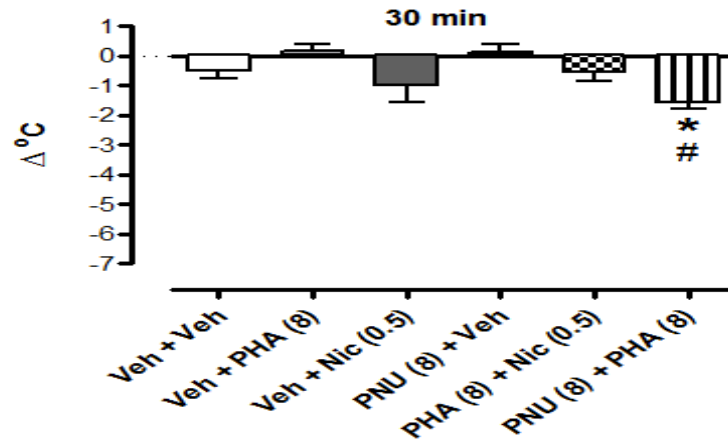
(A)



(B)



(C)



**Figure 27. PNU-120596 enhanced the effect of nicotine-induced hypothermia.**

(A) Effects of different doses of PNU-120596 (1, 4 and 8mg kg<sup>-1</sup>, i.p.) on nicotine-induced hypothermia (0.5mg kg<sup>-1</sup>, s.c.). Mice were treated with either vehicle or PNU-120596 15min before vehicle or nicotine. Body temperatures were measured 30 min after nicotine. (B) The effects of MLA on PNU-120596 enhanced nicotine-induced hypothermia. Mice were treated with vehicle or MLA (10mg kg<sup>-1</sup>, s.c.) 15 min before PNU-120596 (8mg kg<sup>-1</sup>, s.c.). Mice were then injected with nicotine (0.5mg kg<sup>-1</sup>, s.c.) 15 min after PNU-120596 or vehicle. Changes in body temperature were measured 30 min later. (C) Interaction of PHA-543613 (8mg kg<sup>-1</sup>, s.c.) with nicotine (0.5mg kg<sup>-1</sup>, s.c.) or PNU-120596 (8mg kg<sup>-1</sup>, i.p.). Mice were treated with vehicle, PNU-120596 or PHA-543613 15 min before vehicle, PHA-543613 or nicotine. Body temperatures were measured 30 min after the first drug. Each point represents the mean ± SEM of 6 mice. The asterisks denote the significance levels when compared with the control group: \*p < 0.05.

### 3.4 Summary of Chapter III Results

Characterizing PNU and choline separately, we found that both PNU and choline dose-dependently reduced formalin-induced pain behaviors in both phases of the formalin test. The ED<sub>50</sub> (95% confidence limits) values PNU-120596 and choline in phase II were 2.61 (2.13-3.19) and 18.90 (14.79-24.17) mg/kg. Subsequently, evaluating the interaction of PNU-120596 with choline, we showed a leftward shift in the dose-response curve of choline. An isobolographic analysis, paired with a comparison of predicted and experimentally-derived ED<sub>50</sub> values, confirmed the synergistic nature of the interaction between PNU-120596 and choline. Furthermore, PNU-120596 was found to enhance PHA-543613's and nicotine's, but not morphine's, effects in the formalin test.

Additionally, PNU-120596 did not significantly affect the nicotine dose-response curve with respect to nicotine-induced decreased locomotor activity or seizures. In contrast, PNU interacting with lowest dose of nicotine (0.5 mg/kg) induced a significant hypothermic effect. Moreover, this enhancement was blocked by the  $\alpha 7$  nAChR antagonist MLA, suggesting that PNU-120596-enhancement of nicotine-induced hypothermia was  $\alpha 7$  nAChR-mediated. Lastly, the interaction of PNU-120596 and PHA-543613, compared to PHA-543613 or vehicle alone, significantly enhanced hypothermia, while PHA-543613 did not enhance nicotine-induced hypothermia.



## CHAPTER IV

### Effects of Alpha 7 Positive Allosteric Modulators in Murine Inflammatory and Chronic Neuropathic Pain Models

#### 4.1 Chapter IV Introduction

Chronic neuropathic pain arguably arises due to long-term plasticity changes in somatosensory pathways from the periphery to the cortex. These plasticity changes often occur after nerve injury and/or dysfunction in the CNS, resulting in significantly enhanced pain sensation (hyperalgesia) or in otherwise non-noxious stimuli to cause pain (allodynia) (Wang et al., 2011; Zhuo, 2007 and Harden, 2005). Increased pain sensitivity, one of the most common signs of an inflammatory disorder, is mediated by a host of different factors, including enzymes, neuropeptides, eicosanoids, chemokines and cytokines (Dray and Bevan, 1993; Wang et al., 2011)

Previous studies suggest utility of nAChR agonists to treat chronic pain conditions (Vincler, 2005; Khan et al., 2003; Miao et al., 2004). Recent work has focused on the role of the  $\alpha 7$  receptors in modulating inflammation and nociception (Westman et al., 2010; Marrero and Bencherif, 2009; Medhurst et al., 2008). In addition to their neuronal presence,  $\alpha 7$  receptors are expressed on macrophages (Tracy et al., 2002; Wang and Wang, 2003; Ulloa et al., 2005), which are key immune cells involved in the initiation, maintenance, and resolution of inflammation (Fujiwara and Kobayashi, 2005). Previous studies have demonstrated the importance of ACh directly interacting with  $\alpha 7$  nAChRs expressed on macrophages and other cytokine-producing cells in down-regulating proinflammatory cytokine synthesis and preventing tissue damage (Tracey, 2002; Wang et al., 2009). In addition, Xiao et al. (2002) showed an up-regulation of  $\alpha 7$

subunit expression in the rat dorsal root ganglion fourteen days after sciatic nerve axotomy. Moreover,  $\alpha 7$  agonists elicited significant anti-inflammatory and antinociceptive effects in rodent models of chronic neuropathic pain and inflammation (Gurun et al., 2009; Wang et al., 2005; Medhurst et al., 2008; Hamurtekin and Gurun, 2006; Damaj et al., 2000). Consequently, the  $\alpha 7$  nAChR represents a promising target for the development of analgesic and anti-inflammatory agents.

Thus, in the present study, we evaluated whether potentiating the endogenous  $\alpha 7$  cholinergic system through the allosteric modulation of  $\alpha 7$  nAChRs produces anti-inflammatory, anti-hyperalgesic and anti-allodynic effects in mouse models of inflammation and chronic neuropathic pain. Accordingly, NS1738 (a type I  $\alpha 7$  nAChR PAM) and PNU-120596 (a type II  $\alpha 7$  nAChR PAM) were evaluated in the carrageenan short-term inflammatory pain and the CCI neuropathic pain models. In addition, we evaluated whether PNU-120596 enhances the antinociceptive effects of a selective  $\alpha 7$  nAChRs agonist, PHA-543611, in these models.

## **4.2 Materials and Methods**

### **4.2.2 Animals**

Naïve male adult ICR (Harlan Laboratories; Indianapolis, IN) and C57BL/6J (Jackson Laboratories; Bar Harbor, ME) mice weighing between 20 and 30 g served as subjects. All other animal parameters including housing, care and demographics were the same as described in chapter II.

### 4.2.3 Drugs

MLA was purchased from RBI Natick, MA). PNU 120596 [1-(5-Chloro-2,4-dimethoxyphenyl)-3-(5-methyl-isoxazol-3-yl)] and PHA-543613 were obtained from the National Institute on Drug Abuse (NIDA) supply program. NS 1738 [N-(5-Chloro-2-hydroxyphenyl)-N'-[2-chloro-5-(trifluoromethyl)phenyl] was purchased from Tocris Biosciences (Minneapolis, MN). All drugs except for PNU-120596 and NS1738 were dissolved in physiological saline (0.9% sodium chloride) and injected subcutaneously (s.c.) in a volume of 1ml/100 g body weight unless noted otherwise. PNU 120596 and NS1738 were dissolved in vehicle consisting of 1 volume ethanol, 1 volume Emulphor-620 (Rhone-Poulenc, Inc., Princeton, NJ) and 18 volumes distilled water, and injected via the (i.p.) route of administration. Doses of NS1738 and PNU-120596 were chosen based on their activity in *in vivo* models of memory and cognition (Timmermann et al., 2007; Christensen et al., 2010). Lambda-carrageenan was purchased from Sigma-Aldrich and dissolved in saline. All doses are expressed as the free base of the drug.

### 4.2.4 Intrathecal Injections

Injections were administered as described in chapter II.

### 4.2.5 Prolonged Pain Models

#### 4.2.5.1 *Carrageenan Model of Short Inflammatory Pain*

These procedures have been previously described by (Cravatt et al., 2004; Lichtman et al., 2004). Briefly, edema was induced by giving an intraplantar injection of 0.5% lambda-carrageenan in a 20  $\mu$ l volume into the hind right paw using a 30<sup>1/2</sup> gauge needle. Saline (20  $\mu$ l) was injected into the left hind paw.

Carrageenan-induced inflammatory pain was tested by measuring paw edema. The thickness of the carrageenan-treated and control paws were measured both before and after carrageenan injection at the time points indicated in Results, using digital calipers (Traceable Calipers, Friendswood, TX). Data were recorded to the nearest  $\pm 0.01$ mm and expressed as change in paw thickness  $\Delta PT = \text{right paw thickness} - \text{left paw thickness}$ .

Thermal hyperalgesia was also used to measure carrageenan-induced inflammatory pain. Mice were placed in clear plastic chambers (7 cm x 9 cm x 10 cm) on an elevated glass surface and allowed to acclimatize for at least 30 min before testing. The infrared beam of a radiant heat source was directed at the plantar surface of each hind paw, in the area immediately proximal to the toes. A 20-second cut-off time was used. Three measures of paw withdrawal latency were taken and averaged for each hind-paw using the Hargreaves test (Yalcin et al., 2011). The paw withdrawal latency was defined as the time from the onset of radiant heat to withdrawal of the animal's hind paw (Lichtman et al., 2004). Withdrawal thresholds were measured in each hind paw. Results were expressed as  $\Delta PWL \text{ (sec)} = \text{ipsilateral latency} - \text{contralateral latency}$ . Paw edema and thermal hyperalgesia were both used in the following protocols.

#### Determining the effects of NS1738 and PNU-120596 on developing inflammation after carrageenan injection

NS1738 or vehicle was administered 15 min prior to an i.pl. injection of carrageenan and then mice were tested 6 h after the injection, whereas for PNU-120596, drug was injected 15 min prior to an i.pl. injection of carrageenan and then mice were tested 15 min and 1, 6 and 24 h after the carrageenan injection. For the antagonist study, MLA was injected via i.t. 5 min or s.c. 10 min prior to a PNU-120596 injection, which was followed 15 min later with carrageenan and

then mice were tested 6 h after the last injection.

#### Effect of PNU-120596 on reversing carrageenan-induced thermal hyperalgesia and paw edema

To determine whether PNU-120596 can reduce established thermal hyperalgesia and paw edema after carrageenan injection, paw withdrawal latency and paw diameter were measured after the establishment of thermal hyperalgesia or paw edema, respectively (3 h after carrageenan administration). Subsequently, a treatment of either vehicle or PNU-120596 (8mg/kg, i.p.) was administered 3 h after carrageenan, and paw withdrawal latency and paw diameter were measured 15 min, 1 h, and 3 h after either treatment.

#### 4.2.5.2 *Chronic Constriction Injury (CCI)*

Mice were anesthetized with pentobarbital (45 mg/kg, i.p.). An incision was made just below the hipbone, parallel to the sciatic nerve. The right common sciatic nerve was exposed at the level proximal to the sciatic trifurcation, and a nerve segment 3-5 mm long was separated from surrounding connective tissue. Two tight ligatures of 6-0 silk suture, spaced 1.0-1.5 mm apart, were made around the nerve. Skin and muscles were closed with suture. This procedure resulted in chronic constrictive injury of the ligated nerve. In sham-operated controls, an identical surgical incision was performed on the same paw, except that the sciatic nerve was not ligated. For the purposes of this paper, the paw that underwent surgery will be referred to as the ipsilateral paw, and the paw that did not undergo surgery will be referred to as the contralateral paw. After surgery, mice were allowed to recover in a warmed cage on clean paper towels and then returned to their home cage after regaining consciousness. Any suture that remained after two weeks was removed from the healed surgical wound. We assessed both thermal hyperalgesia and mechanical allodynia in CCI mice via the Hargreaves test and von Frey filaments test,

respectively.

Mechanical allodynia thresholds were determined according to the method of Chaplan et al. (1994). Mice were placed in a Plexiglas cage with mesh metal flooring and allowed to acclimate for 30 min before testing. A series of calibrated von Frey filaments (Stoelting, Wood Dale, IL) with logarithmically incremental stiffness ranging from 2.83 to 5.88 expressed  $\text{dsLog}_{10}$  of [10  $\times$  force in (mg)] were applied to the paw with a modified up-down method (Dixon, 1965). In the absence of a paw withdrawal response to the initially selected filament, a thicker thicker corresponding to a stronger stimulus was presented. In the event of paw withdrawal, the next weaker stimulus was chosen. Each hair was presented perpendicularly against the paw, with sufficient force to cause slight bending, and held 2–3 s. The stimulation of the same intensity was applied 5 times to the hind paw at intervals of a few seconds. The mechanical threshold was expressed as  $\text{Log}_{10}$  of [10  $\times$  force in (mg)], indicating the force of the Von Frey hair to which the animal reacted (paw withdrawn, licking or shaking).

Thermal hyperalgesia, as measured via the Hargreaves test as described earlier in the context of carrageenan, and mechanical allodynia were used in the following protocols.

#### Determining the Effects of NS1738 and PNU-120596 on CCI-induced Neuropathic Pain

In the CCI model of neuropathic pain, mice were pretreated with either vehicle or NS1738, and then tested 15 min and 1, 3 and 6h later in the Hargreaves plantar stimulator test. The other group of mice was pretreated with vehicle or different doses of PNU-120596 and then tested 15 min and 1, 6 and 24 h later in the Hargreaves plantar stimulator test. In regard to the sham group; mice were pretreated with either vehicle or PNU-120596 and then tested 3 h later in the Hargreaves plantar stimulator test. For the antagonist study, mice were pretreated with MLA

i.p and then, 10 min later, were administered PNU-120596, and were tested in the Hargreaves plantar stimulator test 15 min later.

#### Determining the Effects of NS1738 and PNU-120596 on CCI-induced Mechanical Allodynia

Mechanical stimuli thresholds were determined for each animal after 15min, 3 and 6 h after the injection of NS1738 or PNU-120596 in the mice that had CCI surgery. To determine whether PNU-120596 enhances the effects of the selective  $\alpha 7$  agonist PHA-543613, PHA-543613-treated mice were given a 15 min pretreatment of PNU-120596 and were tested 30 min, 3 and 6 h after the PHA-543613 injection.

#### 4.2.6 Locomotor Activity

Mice were placed into individual Omnitech photocell activity cages (28 x 16.5 cm) after 15 min after the i.p. administration of vehicle, NS1738 or PNU-120596 at different doses. Interruptions of the photocell beams (two banks of eight cells each), which assess walking and rearing, were then recorded for the next 30 min. Data were expressed as the number of photocell interruptions.

#### 4.2.7 Motor Coordination

We also evaluated the effect of PNU-120596, at doses that produce antinociceptive effects (8mg/kg, i.p.), in the rotarod test. In order to measure motor coordination, we used Rotarod (IITC Inc. Life Science). The animals are placed on textured drums (1¼ inch diameter) to avoid slipping. When an animal falls onto the individual sensing platforms, test results are recorded. Five mice were tested at a time using a rate of 4 rpm. Naive mice were trained until they remained on the rotarod for 5 min. Animals that failed to meet this criterion within three

trials were discarded. Fifteen min after the injection of vehicle or drugs, mice were placed on the rotarod for 5 min. If a mouse fell from the rotarod during this time period, it was scored as motor impaired. Percent impairment was calculated as follows: % impairment = [(180-test time/180\*100)]. Mice were pretreated with either i.p. vehicle or PNU-120596 15 min before the test.

#### 4.2.8 Statistical Analysis

The data obtained were analyzed using a GraphPad software program and expressed as the mean  $\pm$  S.E.M. Statistical analyses were done using student's t test and one-way or repeated measures two-way analysis of variance (ANOVA) tests. Tukey's or Bonferroni's tests were used for post hoc analysis. *P*-values less than 0.05 ( $p < 0.05$ ) were considered significant.

### 4.3 Results

#### 4.3.1 Effects of NS1738 and PNU-120596 on Developing Inflammation After Carrageenan Injection

Mice were given an i.pl. injection of carrageenan, then tested for hyperalgesia and edema 6 h later. Anti-hyperalgesic and anti-edematous effects of the  $\alpha 7$  nAChRs PAMs 15 min after i.p. administration were determined. The administration of 10 and 30 mg/kg of NS1738 15 min before i.pl. carrageenan blocked the development of hyperalgesic responses [ $F_{(2,27)} = 9.07$ ,  $p < 0.01$ ] (Figure 28A). However, at these doses NS1738 did not diminish the magnitude of paw edema [ $F_{(2,27)} = 0.05$ ,  $p = 0.95$ ] (Figure 28B).

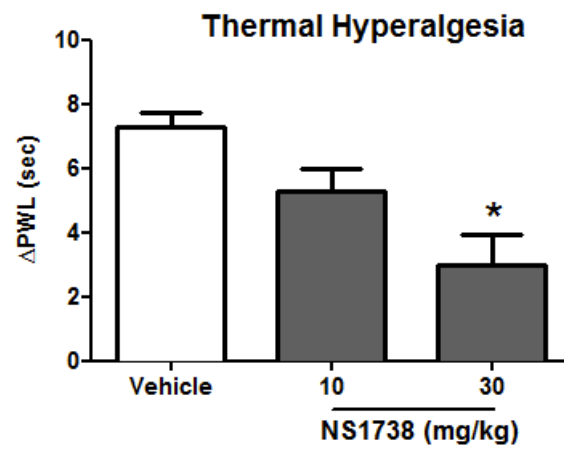
In contrast, administration of PNU-120596 15 min before an i.pl. carrageenan injection blocked hyperalgesic responses [ $F_{(2,15)} = 17.26$ ,  $p < 0.001$ ] (Figure 28C) and significantly



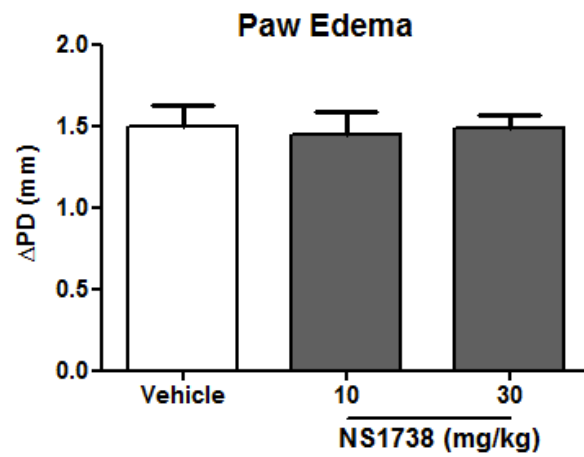
diminished the magnitude of paw edema [ $F_{(2,15)} = 20.46, p < 0.001$ ] (Figure 28D) at 6 h after i.p. carrageenan administration.

We next evaluated whether inhibition of  $\alpha 7$  nAChRs would block PNU-120596's effects on hyperalgesia and paw edema. A single s.c. injection of MLA (10mg/kg) given 10 min before i.p. PNU-120596 completely blocked the anti-hyperalgesic effects of PNU-120596 [ $F_{(3,36)} = 35.87, p < 0.001$ ] (Figure 29A). Similarly, MLA given 10 min before PNU-120596 completely antagonized PNU-120596 anti-edematous effects [ $F_{(3,36)} = 13.35, p < 0.001$ ] (Figure 29B). MLA (10  $\mu$ g/mouse, i.t.), given 5 min before PNU-120596, also completely blocked PNU-120596's anti-hyperalgesic [ $F_{(3,36)} = 12.84, p < 0.001$ ] and anti-edematous [ $F_{(3,36)} = 11.44, p < 0.001$ ] effects (Figure 29C & D).

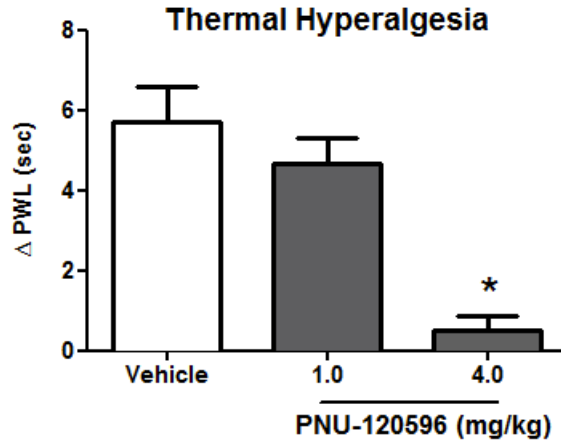
(A)



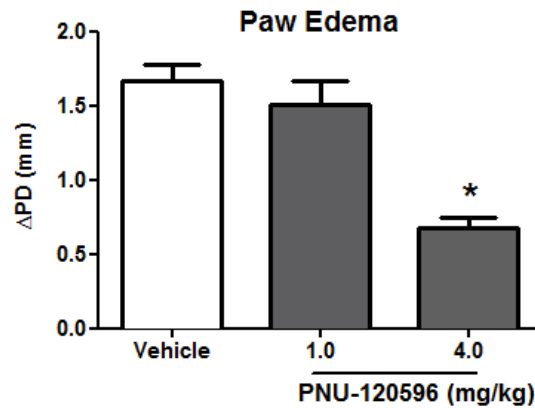
(B)



(C)



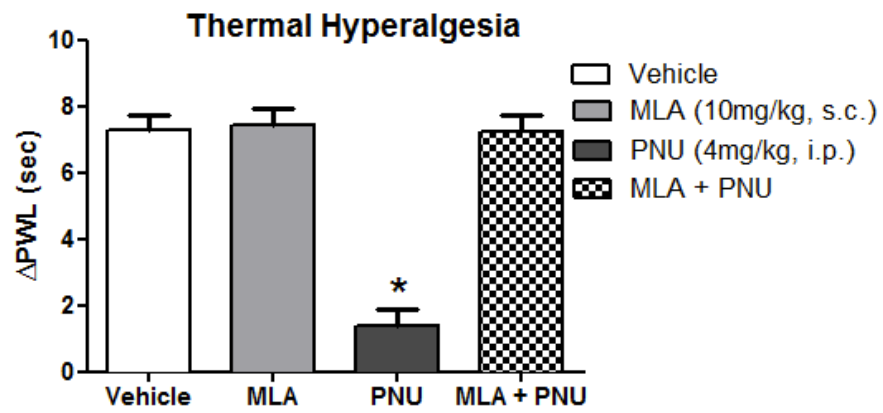
(D)



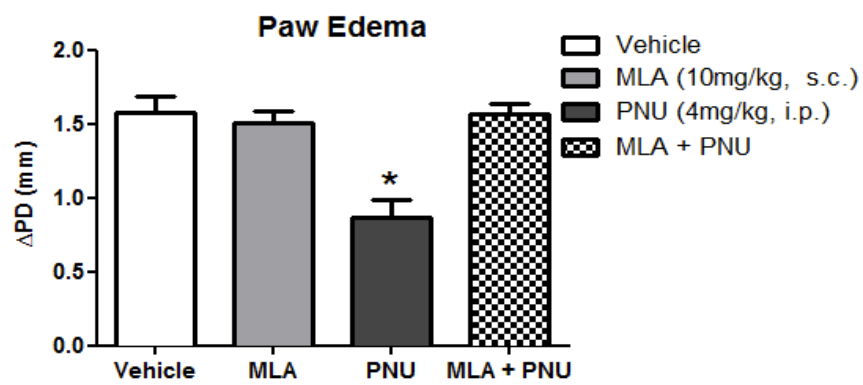
**Figure 28. Carrageenan-induced hyperalgesia was reduced by NS1738 while both carrageenan-induced hyperalgesia and edema were reduced by PNU-120596.**

Mice were given an i.pl. injection of carrageenan, and hyperalgesia and paw-edema were assessed 6 hours later. (A) NS1738 (30mg/kg) at 15 min before carrageenan injection significantly reduced hyperalgesia, but not paw-edema (B). An i.p. injection of PNU-120596 (4mg/kg) significantly reduced thermal hyperalgesia and paw edema 6 hours after a carrageenan injection (C & D), respectively. Baseline values for PWL and paw thickness were 11.5 s and 4.06 mm in carrageenan-injected paws, respectively. Baseline values for control paws were 12.1 s (PWL) and 2.15 mm (paw thickness). \*,  $p < 0.05$  when compared to mice that were given an i.pl. injection of carrageenan and an i.p. injection of vehicle. N= six mice per group. Data are expressed as means  $\pm$  S.E.M.

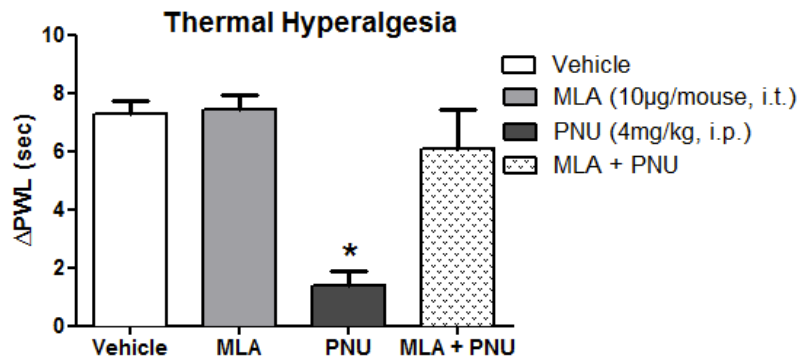
(A)



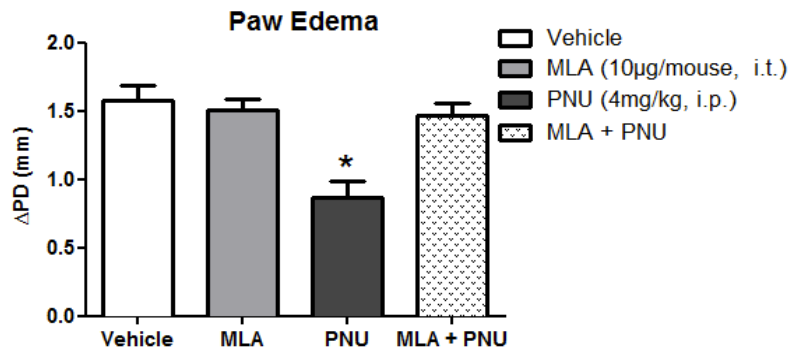
(B)



(C)



(D)



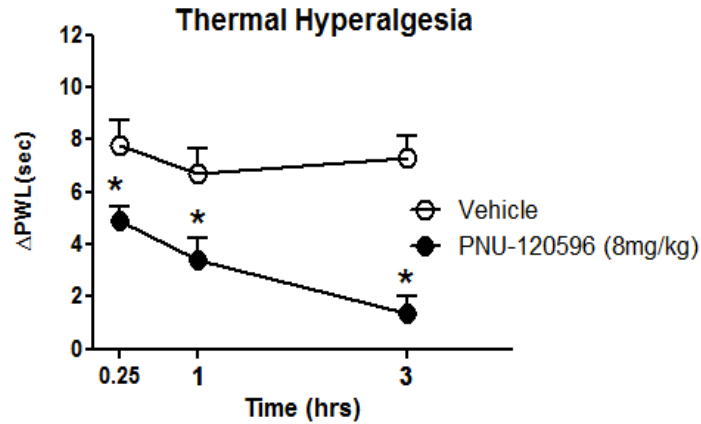
**Figure 29. Effects of a single injection of the  $\alpha 7$  nAChR antagonist MLA (s.c.) or (i.t.) on PNU-120596's anti-hyperalgesic and anti-edematous effects.**

Mice were given an i.pl. injection of carrageenan, after which hyperalgesia and paw edema were assessed (6 h later). PNU-120596 (4mg/kg), at 15 min before the carrageenan injection, significantly attenuated carrageenan-induced hyperalgesia. This anti-hyperalgesic effect was blocked by a pretreatment of  $\alpha 7$  nAChR antagonist MLA (10mg/kg) 10 min before PNU-120596 administration (A). PNU-120596 (4mg/kg) administration significantly attenuated carrageenan-induced paw edema, and this effect was blocked by 10 min pretreatment with MLA, (10mg/kg) before PNU-120596 (B). PNU-120596's anti-hyperalgesic effect was also blocked by a pretreatment of  $\alpha 7$  nAChR antagonist MLA (10 $\mu$ g/mouse) 5 min before PNU-120596 administration (C). PNU-120596 (4mg/kg) administration also significantly attenuated carrageenan-induced paw edema, and this effect was blocked by 5 min pretreatment with MLA, (10 $\mu$ g/mouse) before PNU-120596 (D).\*,  $p < 0.05$  when compared to mice that were given an i.pl. injection of carrageenan and an i.t. injection of vehicle. N= six mice per group. Data are expressed as means  $\pm$  S.E.M.

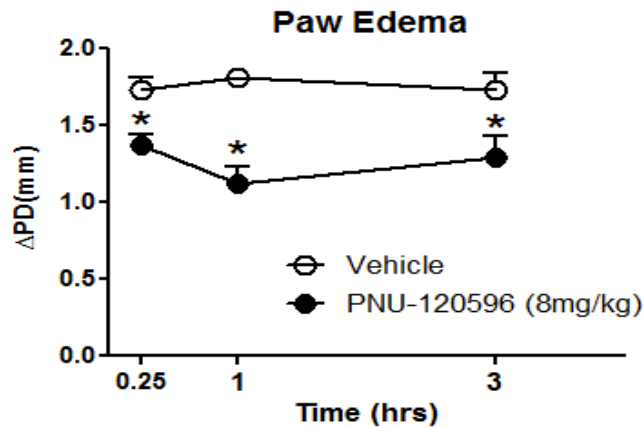
### 4.3.2 Effect of PNU-120596 on Reversing Carrageenan-induced Thermal Hyperalgesia and Paw Edema

We then addressed the clinically relevant question of whether PNU-120506 can reverse carrageenan-induced thermal hyperalgesia and paw edema after they have been established. This is a particularly important question as anti-inflammatory agents are most often administered after inflammation has already occurred. Mice were administered i.pl. carrageenan and then, 3 h later, administered PNU-120596 (i.p.). Subsequently, mice were tested for thermal hyperalgesia and for paw thickness, at 15 min, 1 h and 3 h post-PNU-120596 treatment. As shown in Figure 30A, PNU-120596 reversed carrageen-induced thermal hyperalgesia at 15 min [ $t_{(10)} = 2.542$ ,  $p < 0.05$ ], 1 h [ $t_{(10)} = 2.58$ ,  $p < 0.05$ ] and 3 h [ $t_{(10)} = 5.36$ ,  $p < 0.001$ ] after the carrageenan injection. Likewise, as shown (Figure 30B) PNU-120596 attenuated carrageenan-induced paw edema at 15 min [ $t_{(10)} = 3.10$ ,  $p < 0.05$ ], 1h [ $t_{(10)} = 5.55$ ,  $p < 0.001$ ] and 3h [ $t_{(10)} = 2.47$ ,  $p < 0.05$ ] after the establishment of carrageenan (i.pl.)-induced thermal hyperalgesia.

(A)



(B)



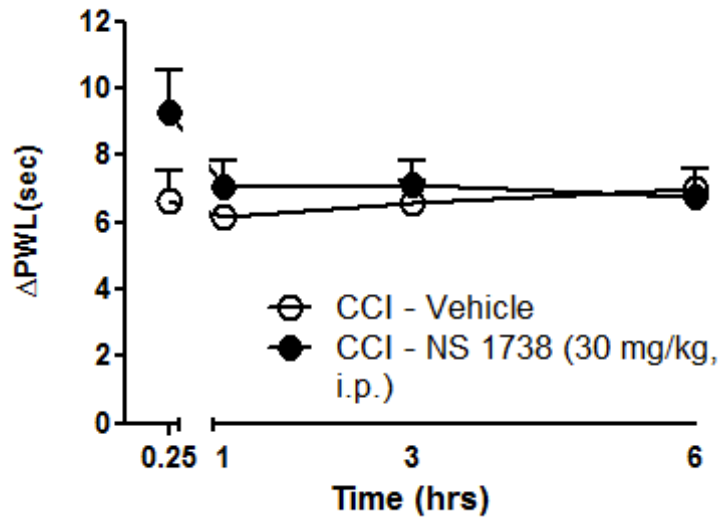
**Figure 30. Effect of PNU-120596 (8mg/kg, i.p) on established thermal hyperalgesia and paw edema after carrageenan injection.**

Thermal hyperalgesia and paw diameter was measured prior to and 3 h after carrageenan administration. Samples sizes were 6 mice per group. The mean  $\pm$  S.E.M for the carrageenan-administered thermal hyperalgesia group was  $7 \pm 0.7$  for the vehicle group and  $5.1 \pm 0.7$  for the group that received PNU-120596. Paw thickness means  $\pm$  S.E.M were  $1.7 \pm 0.1$  for the vehicle group and  $1.4 \pm 0.1$  for the PNU-120596-treated group. (A) PNU-120596 significantly attenuated carrageenan-induced thermal hyperalgesia at 15 min, 1 and 3h after the establishment of carrageenan-induced thermal hyperalgesia. (B) Effects of PNU-120596 on carrageenan-induced paw edema. PNU-120596 significantly reduced paw edema. PNU-120596 was administered 3 h after carrageenan was injected into the paw, then thermal hyperalgesia and paw diameter were measured after 15 min, 1 and 3 h following PNU-120596 injection. \*,  $p < 0.05$  when compared to mice that had a right sciatic nerve constriction injury and were administered i.p. vehicle. N = six mice per group. Data are expressed as means  $\pm$  S.E.M.

#### 4.3.3 Effect of NS1738 on Thermal Hyperalgesia in the CCI Model

In the CCI model of neuropathic pain, NS1738 (30mg/kg) did not attenuate thermal hyperalgesia at any time point tested (Figure 31). Ten days after CCI surgery, change in paw withdrawal latencies ( $\Delta$ PWL) in the NS1738-treated CCI group were not significantly different than thresholds recorded from the vehicle-treated CCI group [ $F_{(3,4)} = 0.69$ ,  $p = 0.60$ ].





**Figure 31. NS1738 did not attenuate chronic constriction injury (CCI)-induced thermal hyperalgesia.**

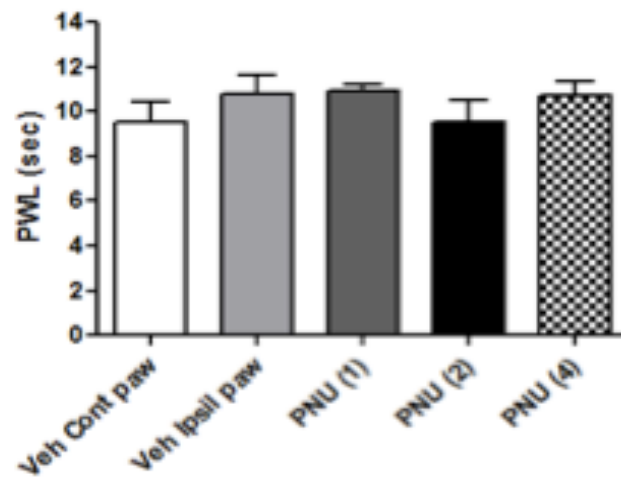
Ten days after chronic constriction injury, mice were pretreated with NS1738, and then tested 15 min later in the Hargreaves plantar stimulator test. NS1738 (30mg/kg, i.p.) did not attenuate CCI-induced hyperalgesia. Data from paws ipsilateral to nerve injury are shown. Sham group did not differ between drug treatment (data not shown). \*,  $p < 0.05$  when compared to mice that had a right sciatic nerve constriction injury and were administered i.p. vehicle. N= six mice per group. Data are expressed as means  $\pm$  S.E.M.

#### 4.3.4 Effects of PNU-120596 on thermal hyperalgesia in the CCI Mice

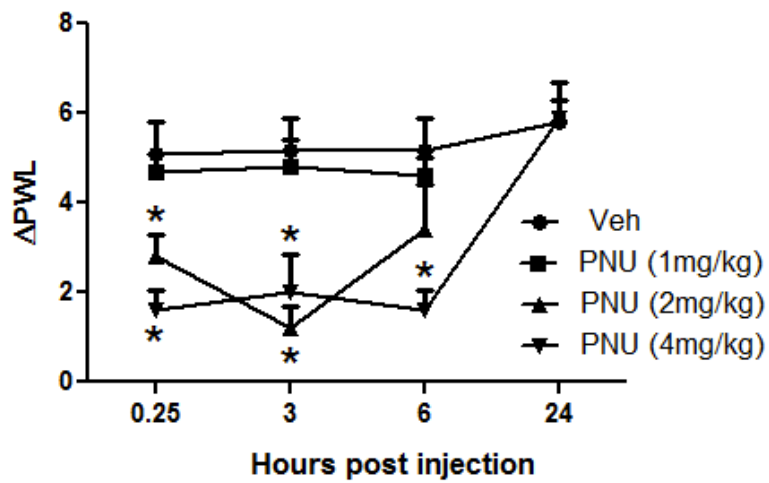
As shown in Figure 32A, PNU-120596 did not produce significant [ $F_{(3,20)} = 0.84$ ,  $p = 0.49$ ] differences in thermal sensitivity in control mice. The effects of PNU-120596 (1, 2 and 4 mg/kg, i.p.) on the thermal sensitivity in sham mice group were measured 3 h after injection. In contrast, PNU-120596 dose-dependently reversed thermal hyperalgesia as shown in Figure 32B. These anti-hyperalgesic effects were seen at 15 min [ $F_{(3,20)} = 9.78$ ,  $p < 0.001$ ], 3 h [ $F_{(3,20)} = 8.28$ ,  $p < 0.001$ ] and 6 h [ $F_{(3,20)} = 5.13$ ,  $p < 0.01$ ], but disappeared 24 h after PNU-120596 administration [ $t(10) = 0.11$ ,  $p = 0.92$ ].

To determine whether the anti-hyperalgesic effects of PNU-120596 were dependent on  $\alpha 7$  nAChRs, mice were administered MLA (10mg/kg, s.c.), an  $\alpha 7$  nAChRs antagonist, 10 min before PNU-120596 and thermal hyperalgesia was measured 15 min after PNU-120596 (4mg/kg, i.p.) administration. As shown in Figure 32C, MLA significantly blocked the anti-hyperalgesic effect of PNU-120596 [ $F_{(2,10)} = 13.35$ ,  $p < 0.01$ ].

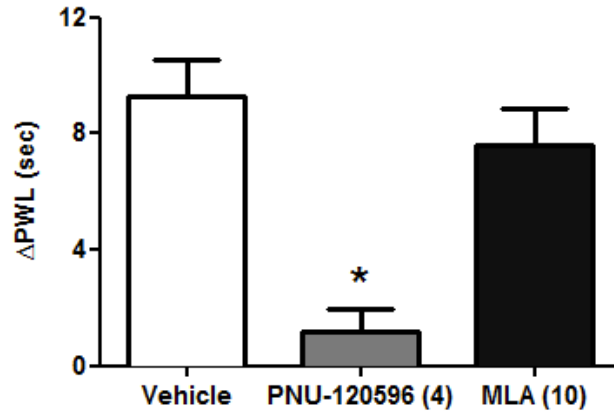
(A)



(B)



(C)



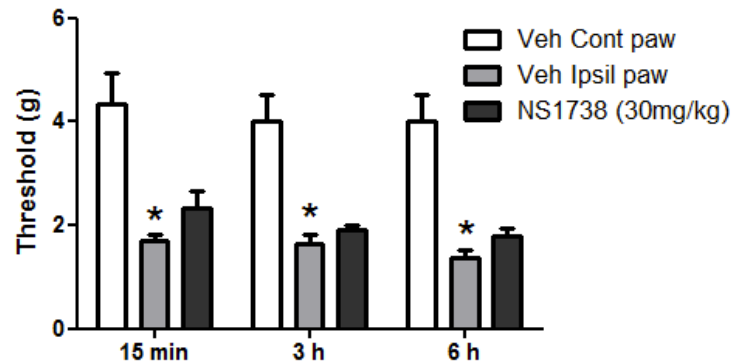
**Figure 32. The anti-hyperalgesic effects of PNU-120596 in CCI-induced thermal hyperalgesia is  $\alpha 7$  nAChR-mediated.**

Mice were subjected to CCI and tested for thermal hyperalgesia. Mice were treated with PNU-120596 (1, 2 and 4 mg/kg, i.p.) and thermal hyperalgesia was measured at 15 min, 3, 6 and 24 h post-treatment with PNU-120596. (A) Sham group did not differ between drug treatment. (B) PNU-120596 reduced the intensity of thermal hyperalgesia in a dose- and time-related manner. (C) MLA, the  $\alpha 7$  nAChR antagonist completely blocked the anti-hyperalgesic effects of PNU-120596. Data from paws ipsilateral to nerve injury are shown. \*,  $p < 0.05$  when compared to mice that had a right sciatic nerve constriction injury and were administered i.p. vehicle. N= six mice per group. Data are expressed as means  $\pm$  S.E.M.

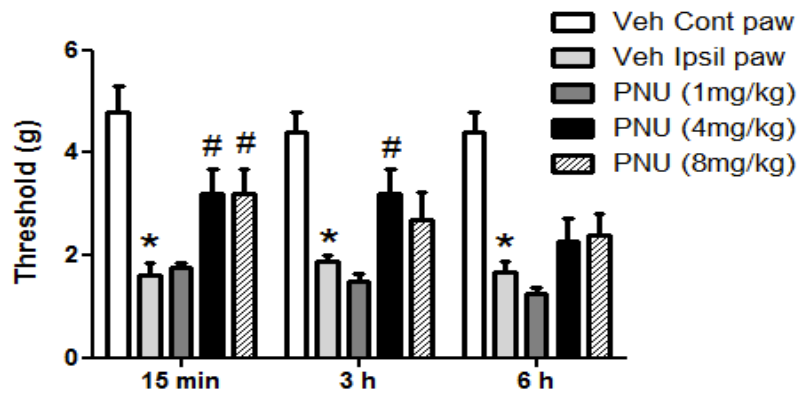
#### 4.3.5 Effects of NS1738 and PNU-120596 on Mechanical Allodynia in CCI Mice

Fifteen days after the CCI surgery, mice were given an i.p. injection of NS1738, PNU-120596, or vehicle. In mice that received vehicle or NS1738, ipsilateral paws displayed significantly lower thresholds than contralateral paws after 15 min [ $t_{(10)} = 4.19$ ,  $p < 0.01$ ], 3 h [ $t_{(10)} = 4.34$ ,  $p < 0.01$ ] and 6 h [ $t_{(10)} = 4.90$ ,  $p < 0.001$ ] as shown in Figure 33A. NS1738 (30mg/kg i.p.) treatment did not significantly reverse mechanical allodynia thresholds produced by a vehicle (i.p.) treatment at 15 min [ $t_{(10)} = 1.76$ ,  $p = 0.11$ ], 3 h [ $t_{(10)} = 1.33$ ,  $p = 0.21$ ], or 6 h [ $t_{(10)} = 2.21$ ,  $p = 0.05$ ] (Figure 33A). In mice that received vehicle or PNU-120596, ipsilateral paws displayed significantly lower thresholds than contralateral paws after at 15 min [ $t_{(8)} = 5.84$ ,  $p < 0.001$ ], 3 h [ $t_{(8)} = 6.03$ ,  $p < 0.001$ ] and 6 h [ $t_{(8)} = 6.05$ ,  $p < 0.001$ ] as shown (Figure 33B). In contrast to NS1738, i.p. PNU-120596 dose-dependently produced significant anti-allodynic effects [ $F_{(3,17)} = 6.15$ ,  $p < 0.01$ ], compared to those produced by an i.p. vehicle treatment, when tested 15 min post-treatment (Figure 33B). The anti-allodynic effects of PNU-120596 (4 mg/kg) persisted for 3 h but no longer significantly differed from vehicle at 6h [ $F_{(3,17)} = 3.20$ ,  $p = 0.05$ ] (Figure 33B). Thresholds produced by PNU-120596-treated mice in the sham group (the control) did not differ from those produced by vehicle-treated mice (data not shown).

(A)



(B)

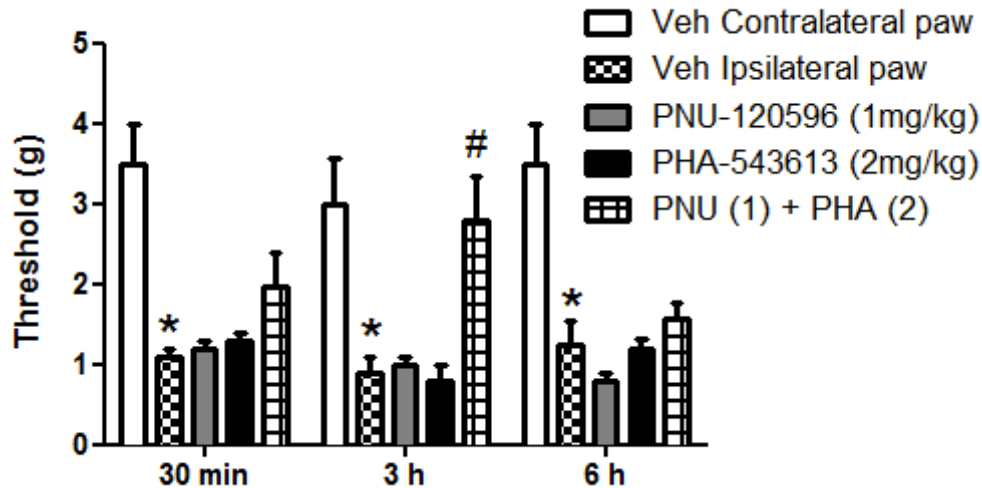


**Figure 33. CCI-induced mechanical allodynia was attenuated by PNU-120596 at higher doses and at times closer to treatment, but not by NS1738 at any dose or time.**

Two groups of CCI mice, one pretreated with NS1738 or vehicle and one pretreated with PNU-120596 or vehicle, were tested at 15 min, 3 h, and 6 h after treatment in the mechanical allodynia test. (A) NS1738 (30mg/kg, i.p.) did not attenuate CCI-induced allodynia at 15 min, 3 h, or 6 h, and allodynia thresholds were significantly different in ipsilateral paws (with CCI) than contralateral paws (without CCI). (B) PNU-120596 significantly attenuated CCI-induced allodynia at 4mg/kg and 8mg/kg, but not 1 mg/kg, 15 min after treatment, at only 4mg/kg at 3 h after treatment, and not at all at 6 h after treatment. PNU-120596 was given i.p to animals and 30 min later; their withdrawal thresholds (g) were measured. Mechanical sensitivity in sham mice did not differ between drug treatments (data not shown). Each point represents the mean  $\pm$  SE of 6 mice. Data are presented as means  $\pm$  S.E.M. of paw withdrawal thresholds. \*,  $p < 0.05$  vs. vehicle contralateral (without CCI) paw ; #,  $p < 0.05$  vs. ipsilateral (CCI surgery) paw withdrawal thresholds. N= six mice per group.

#### 4.3.6 Effects of the Interaction Between PNU-120596 and PHA-543613 on Mechanical Allodynia in CCI Mice

We next tested whether combination of PHA-543613 (2mg/kg s.c.), a selective  $\alpha 7$  agonist, and PNU-120596 (1mg/kg i.p.) would produced enhanced anti-allodynic effects in the CCI model. As depicted, Figure 34 shows thresholds from mice administered both PNU-120596 (1mg/kg, i.p.) and PHA-543613 (2mg/kg) or each compound alone. Low dose of PNU-120596 or PHA-543613 did not significantly block mechanical allodynia at any time point. However, the co-administration of these subthreshold doses of PNU-120596 and PHA-543613 significantly reversed mechanical allodynia at 3 h post-treatment compared to the administration of vehicle then vehicle [ $t_{(8)} = 2.72$ ,  $p < 0.05$ ]. However, no significant anti-allodynic effects were found at 30 min [ $t_{(8)} = 1.57$ ,  $p = 0.16$ ] or 6 h [ $t_{(8)} = 0.91$ ,  $p = 0.39$ ] post-treatment (Figure 34).



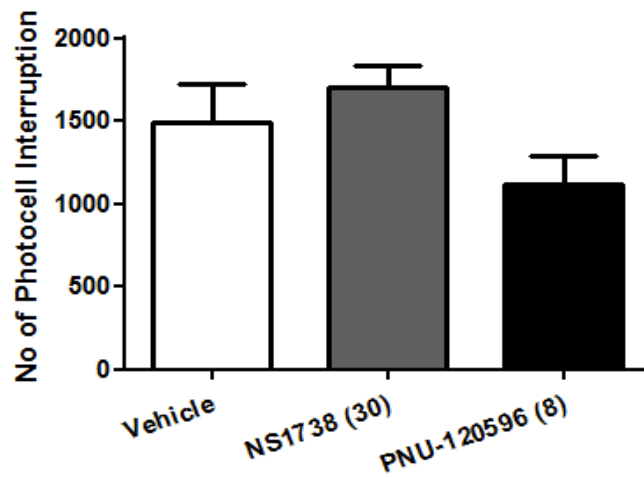
**Figure 34. CCI-induced mechanical allodynia was attenuated 6 h after a PNU-120596, then a PHA-543613, pretreatment (all second treatments were administered 15 min after the first treatment; post-treatment time began after the first treatment).**

Four groups of CCI mice, one pretreated with vehicle (i.p.) then vehicle, one PNU-120596 (1mg/kg, i.p.) then vehicle, one vehicle then PHA-543613 (2mg/kg, s.c.), and one PNU-120596 then PHA-543613 (at the same doses as separately) were tested at 30 min, 3 h, and 6 h after treatment in the mechanical allodynia test. Neither PNU-120596 nor PHA-543613 attenuated CCI-induced allodynia at 30 min, 3 h, or 6 h; allodynia thresholds were significantly different in ipsilateral paws (with CCI) than contralateral paws (without CCI). PNU-120596 pretreatment before PHA-543613 administration significantly attenuated CCI-induced allodynia at the same doses mentioned before at 3 h, but not at 30 min or 6 h, post-treatment. Data are presented as means  $\pm$  S.E.M. of paw withdrawal thresholds. \*,  $p < 0.05$  vs. vehicle contralateral (without CCI) paw ; #,  $p < 0.05$  vs. ipsilateral (CCI surgery) paw withdrawal thresholds. N= 6 mice per group.



#### 4.3.7 Effects of NS1738 and PNU-120596 on Locomotor Activity of Mice

In order to infer whether motor impairment may have contributed to the effects of NS1738 and PNU-120596 in von Frey and Hargreaves tests, we evaluated NS1738 and PNU-120596 on spontaneous activity in mice. As seen in Figure 35, mice treated with an active dose of NS1738 (30mg/kg, i.p.) or PNU-120596 (8 mg/kg, i.p.) did not show significant changes in locomotor activity (locomotor test) [ $F_{(2,10)} = 2.91$ ,  $p = 0.10$ ] 15 min after injection.



**Figure 35. Effects of NS1738 and PNU-120596 on locomotor activity of mice.**

Animals were tested 15 min after injection of vehicle, NS1738 (30mg/kg, i.p.) or PNU-120596 (8mg/kg, i.p.) and their locomotor activity were measured for 30 min. Each point represents 6-8 animals per group.

#### 4.4 Summary of Chapter IV Results

In the carrageenan inflammatory mouse model, we showed that both NS1738 and PNU-120596 blocked the development of thermal hyperalgesic responses, though only PNU-120596 reduced paw edema. These effects were blocked by MLA, suggesting that PNU-120596 reduced carrageenan-induced thermal hyperalgesia and paw edema was  $\alpha 7$  receptor-mediated. Furthermore, PNU-120596 was found to reverse carrageenan-induced thermal hyperalgesia and attenuate carrageenan-induced paw edema after thermal hyperalgesia was established.

In the CCI model, NS1738 did not attenuate thermal hyperalgesia. In sham mice, PNU-120596 produced significant differences in neither thermal sensitivity nor mechanical allodynia. However, it did dose-dependently reverse thermal hyperalgesia lasting up to 24 h. Additionally, PNU-120596's antihyperalgesic effects were antagonized by MLA, suggesting that PNU-120596's effect in the CCI model was  $\alpha 7$  receptor-mediated. Furthermore, PNU-120596 dose-dependently produced anti-allodynic effects in a time-related manner. Indeed, PNU-120596 enhanced PHA-543613's anti-allodynic effect in the CCI model. Finally, as shown in Figure 35, neither NS1738 nor PNU-120596 at antinociceptive doses in the carrageenan and CCI altered locomotor activity in mice.

## CHAPTER V

### 5.1 General Discussion

In the present project, we evaluated the antinociceptive activity of  $\alpha 7$  nAChRs PAMs in mouse models of acute, tonic, inflammatory and chronic neuropathic pain behavior. Our results show that, similar to  $\alpha 7$  agonists,  $\alpha 7$  PAMs are not active in acute thermal pain tests (hot-plate and tail-flick) and mechanical sensitivity tests, after systemic administration. The lack of effect of these  $\alpha 7$  direct and indirect agonists suggests that  $\alpha 7$  nAChRs do not play an important role in acute thermal and mechanical pain modulation.

Another model that we tested was inflammatory tonic pain via the formalin test, which consists of two distinct phases. The first phase (immediately after formalin injection), seems to be caused by the direct effect of formalin on sensory C-fibers. The second phase (starting later after formalin injection), known as the inflammatory phase, is associated with the development of a delayed inflammatory response and spinal dorsal horn sensitization (Abbott et al., 1995; Davidson and Carlton, 1998). Earlier studies have shown that opioids, which are centrally-acting drugs, inhibit both phases of the formalin test, while peripherally-acting drugs, such as NSAIDs and corticosteroids, only inhibit the late phase (Shibata et al., 1989).

Of the two types of  $\alpha 7$  PAMs (type I and type II) that we tested, only the type II  $\alpha 7$  PAM (PNU-120596) attenuated pain behavior in the early and late phases of the formalin test. Indeed, PNU-120596 had a long-lasting antinociceptive effect with a greater potency (6-fold) in the late (inflammatory) phase of the test. In contrast, the type I  $\alpha 7$  PAM (NS1738) failed to exhibit antinociceptive effects in the formalin test after systemic (i.p.) and central (i.t.) administration. The lack of effect after NS1738 administration is probably not due to a poor drug distribution to

the brain after systemic injection, since we find NS1738 in the brain after systemic administration. This is also supported by reports that brain concentrations of NS1738 after i.p. injection (30 mg/kg) yields drug levels to be  $\sim 0.80 \mu\text{M}$ , close to those reported to enhance ACh in expressed  $\alpha 7$  nAChRs (Timmermann et al., 2007). In addition, the concentration of NS1738 in the mouse brain is similar to that seen in the rats after i.p. injection of the same dose of the drug (Timmermann et al., 2007). Similarly, PNU-120596 brain levels of  $\sim 0.25 \mu\text{M}$ , after a dose of 1 mg/kg i.p., fall near the EC50 for potentiating effects of PNU-120596 (EC50  $\sim 0.2$ -1.5  $\mu\text{M}$ ) found in various native and expressed  $\alpha 7$  nAChRs preparations (Kalappa et al, 2010; Gronlien et al., 2007; Hurst et al., 2005; Barron et al., 2009; Gusev and Uteshev, 2010). Our data therefore strongly suggest that, because they are present in the brain at different concentrations after i.p. administration type I and II  $\alpha 7$  nAChR PAMs modulate nociceptive behavior in the formalin test through different mechanisms.

The long-acting effect of PNU-120596 in the formalin test cannot be simply explained by the pharmacokinetic profile of the drug. Indeed as shown in Figure 18, most of the PNU-120596 is eliminated from plasma and brain of the animals 4 hr after injection of the drug. These findings indicate discordance between the pharmacokinetic-pharmacodynamic (Figure 18D) properties of PNU-120596 that may point to a key mechanistic feature of the antinociceptive efficacy associated with  $\alpha 7$  nAChRs positive allosteric modulation. In this regard, PNU-120596's properties are similar to those reported with  $\alpha 7$  nAChR agonists such as ABT-107 (Bitner et al., 2010). PNU-120596 appears to offer prolonged efficacy that may be associated with the activation of signaling pathways leading to lasting secondary functional changes linked to synaptic plasticity. Our results with the MEK inhibitor U-0126 blocking the antinociceptive effect of PNU-120596 suggest that one possible post-receptor signaling mechanism may work

through the extracellular-signal-regulated kinases 1 and 2 (ERK1/2). The ERK1/2 pathway regulates a diverse array of cellular functions, such as cell growth, differentiation and survival that may underlie the synaptic plasticity required for persistent pain processes (Alter et al., 2010). Furthermore, El Kouhen et al. (2009), observed a robust increase in ERK1/2 phosphorylation induced by  $\alpha 7$  agonists in the presence of type II PAM in PC12 cells.

In contrast to the type II  $\alpha 7$  PAM, the selective  $\alpha 7$  agonist PHA-543613 had a very narrow window of antinociceptive effect, as shown by its U-shaped dose-effect curve in the formalin test. While drug distribution and metabolism factors could account for the different dose-response profiles of PHA-543613 and PNU-120596, differences in  $\alpha 7$  nAChRs activation and desensitization properties may play a more important role. Like other  $\alpha 7$  agonists, PHA-543613, induces receptor desensitization after an initial phase of receptor activation. Furthermore, tolerance did not develop following chronic exposure of the type II  $\alpha 7$  PAM. Importantly, no changes were seen in body weight gain after chronic administration of PNU-120596 in mice.

In our studies, MLA, an  $\alpha 7$  antagonist, but not DHBE, a  $\beta 2^*$  antagonist, significantly blocked PNU-120596's antinociceptive effect in the early and late phases of the formalin test. This confirms the specificity of PNU-120596 to the  $\alpha 7$  subtype of nAChRs. Using both pharmacological (i.e. naloxone) and genetic approaches (i.e.  $\alpha 7$  KO mice) we additionally confirm that PNU-120596's effect is mediated by  $\alpha 7$  nAChRs and not by opioid receptors.

Likewise, our data in phase II of the formalin test show a blockade of PNU-120596's effect by NS1738, supporting the idea that type II and I may compete for a common allosteric transmembrane site of the  $\alpha 7$  nAChRs. Supporting this allegation, Collins et al. (2011) recently

showed that both type I PAM NS1738 and type II PAM PNU-120596 bind competitively at a shared or overlapping allosteric transmembrane site on the  $\alpha 7$  nAChR.

We observed that PNU-120596 attenuated pain behavior in the early and late phases of the formalin test, suggesting that PNU-120596 acts both centrally and peripherally to reduce tonic pain. It seems that multiple sites are involved in PNU-120596's antinociceptive effect. Indeed, the data with i.t. and i.pl. MLA provides evidence of both central and peripheral involvement, respectively, of PNU-120596 in the formalin test. For example, spinally-administered MLA blocked the antinociceptive effects of systemic PNU-120596. Additionally, significant antinociceptive activity after i.t. injection of the drug itself was observed. These results support the possibility that the spinal cord is an important site of action for PNU-120596's effects in the formalin test.

Additionally, i.pl. MLA pretreatment blocked the antinociceptive effects of i.p. PNU-120596, further implicating a role for local  $\alpha 7$  nAChRs. In the periphery,  $\alpha 7$ -nAChRs are expressed on T cells, macrophages and other immune cells, which are capable of producing ACh (Fujii et al., 2008; Kawashima and Fujii, 2003). The peripheral efficacy of  $\alpha 7$  PNU-120596 in the setting of inflammation could be a result of a modulation of the release of cytokines by local macrophages. Indeed, an earlier study showed that  $\alpha 7^*$  nAChR expression on macrophages is an essential mediator of inflammation (Wang et al., 2003). Additionally, in the rat hindpaw,  $\alpha 7$  nAChRs have been identified on skin keratinocytes and resident macrophages, but not on peripheral nerve endings (Kurzon et al., 2007).

Subsequently, we evaluated the interaction between the  $\alpha 7$  type II PAM PNU-120596 and various nicotinic agonists in a persistent pain model. For that, we examined the interaction of

choline, an endogenous  $\alpha 7$  nAChR agonist, with PNU-120596 in the formalin model. Using an isobolographic analysis, we were able to show synergism between PNU-120596 and choline, demonstrating that PNU-120596 enhances choline's antinociceptive effect in this model. In an attempt to extrapolate these findings to other  $\alpha 7$  nAChR agonists, we tested and supported the hypothesis that PNU-120596 enhances the effects of a selective  $\alpha 7$  agonist PHA-543613 and nicotine in the formalin test. In contrast, PNU-120596 did not alter the effect of morphine in either phase of the formalin test, implying that opioid receptors were not involved in PNU-120596's enhancement effect in this test.

Generally speaking, PAMs are compounds that facilitate endogenous neurotransmission and/or enhance the efficacy and potency of agonists without directly stimulating the agonist binding sites (Maelicke & Albuquerque, 2000; Albuquerque et al., 2001). This relies on the fact that the effects of PAMs manifest, in principle, only when receptors are activated by the endogenous ligand ACh. The fact that the administration of PNU-120596 on its own produced an antinociceptive response, suggests the presence of a pro-antinociceptive endogenous tone mediated by  $\alpha 7$  nAChRs. Both choline and acetylcholine are possible candidates for PNU-120696's effects. It has been shown that PAMs of the  $\alpha 7$  nAChR act by enhancing the actions of the neurotransmitter ACh (Bertrand and Gopalakrishnan, 2007). However, the rapid clearance of ACh by acetylcholinesterases in the synaptic cleft makes it unlikely that ACh neurotransmission alone is enhanced by PNU-120696 and suggests that endogenous choline in the CNS may play a bigger role. In addition, our data showing the ability of systemic PNU-120696 to enhance spinal choline's antinociception in the formalin test reinforces this claim. Still, the relative contribution of choline and ACh in PNU-120696's effects at peripheral sites (immune cells for example) is not clear.



The isobolographic analysis indicated a supra-additive antinociceptive interaction between PNU-120596 and choline. Indeed, as shown in our results, the experimental ED<sub>50</sub> is located below the additive dose line, indicating that their interaction produces synergistic antinociceptive effect in the formalin test. While our results show that exogenous choline administration in the formalin test produces notable *in vivo* antinociceptive effects, endogenous choline is present in the CSF at a much lower concentration (~10 μM) relative to its potency (EC<sub>50</sub> ~ 0.5-1.5mM) as α7 agonist (Alkondon et al., 1997; Papke and Porter, 2002). Hence, endogenous choline may not be effective in activating native α7\* nAChRs in the absence of a PAM because of choline's low potency and thus narrow therapeutic significance. This notion is consistent with the reported absence of cholinergic synaptic inputs, the absence of exogenous nicotinic agonists, and the findings that innate α7\* nAChRs are not actively sustained or desensitized by the low physiological concentrations of choline (Uteshev et al., 2003). However, recently, Kalappa et al., (2010) and Gusev and Uteshev (2010) showed that, in the presence of PNU-120596, physiological concentrations of choline become effective in the activation of native functional α7\* nAChRs in hippocampal CA1 pyramidal neurons. Interestingly, numerous studies have shown that exogenous choline's antinociceptive effect involves modulation of α7 nAChRs in a variety of pain models (Damaj et al., 2000; Wang et al., 2005; Hamurtekin and Gurun, 2006).

Our results with the α7 nAChR PAM are in line with recently reported α4β2 PAM results. NS-9283, an α4β2 selective PAM enhanced the effects of an α4β2 agonist (ABT-594) in rodent inflammatory and chronic neuropathic pain models without exacerbating the agonists' side effects, such as nausea and emesis (Zhu et al., 2011; Lee et al., 2011). It is important to note that in contrast to PNU-120596, the α4β2 PAM failed to produce an antinociceptive in these

models on its own. These differences suggest that in contrast to  $\alpha 7$  nAChRs, tonic endogenous anti-inflammatory and antinociceptive mechanisms mediated by  $\alpha 4\beta 2^*$  nAChRs are not evident. Additionally, the way that PNU-120596 modulates the  $\alpha 7$  nAChRs may play a key role in explaining PNU-120596's antinociceptive effects. As mentioned earlier, PNU-120596 slows additional desensitization of the  $\alpha 7$  nAChRs (Gronlien et al., 2007), therefore, PNU-120596 may have greater effect on  $\alpha 7$  nAChRs activation and desensitization than the  $\alpha 4\beta 2$  nAChR PAM (NS-9283) has on  $\alpha 4\beta 2$  nAChRs.

PNU-120596 failed to alter nicotine-induced seizures, -decrease in motor activity and -antinociception in acute thermal pain tests. Surprisingly, PNU-120596 did significantly enhance nicotine-induced hypothermia via  $\alpha 7$  nAChRs. Since PNU-120596 enhanced the antinociceptive effects of nicotine in the tonic pain model, we subsequently evaluated the interaction between PNU-120596 and nicotine in thermal acute pain models (tail-flick and hot-plate tests). Surprisingly, PNU-120596 did not enhance or block nicotine's effect in both tests. There are several factors that may account for this variability, such as differences between the nociceptive response of the acute thermal pain models, which is based on a short, high intensity stimulus generating a brief pain, and the nociceptive responses of chronic pain models, such as the formalin test, which involves a continued pain generated by injured tissue caused by the i.pl. injection of formalin (Tjolsen et al., 1992). Thus, PNU-120596's modulation of nociception may differ for pain elicited by short- than for pain elicited by long-lasting stimuli.

Furthermore, PNU-120596 produced a greater antinociceptive response in the late phase, which seems to be related to the inflammatory response elicited by formalin (Hunnskaar and Hole, 1987), then in the early phase of the formalin test, suggesting that PNU-120596's antinociceptive

effects might suppress monocyte/macrophage release of inflammatory cytokines. This supports the assertion of  $\alpha 7$  nAChR's role in the cholinergic anti-inflammatory pathway, in which  $\alpha 7$  nAChRs have been shown to be located on macrophages (Tracy, 2002, Wang et al., 2003, Ulloa, 2005 and de Jonge and Ulloa, 2007), lymphocytes (De Rosa et al., 2009) as well as glial cells (microglia, astrocytes) (Wang et al., 2007). PNU-120596's modulation of this  $\alpha 7$  nAChR through ACh and/or choline, along with prolonged receptor desensitization associated with a high calcium influx, may stop nuclear factor  $\kappa B$  (NF- $\kappa B$ ) stimulation (Tracey et al., 2005; Babaev et al., 2000).

The fact that PNU-120596 enhanced nicotine's effects in the formalin test lead us to evaluate if it would potentiate other nicotinic responses, in particular those associated with CNS-related adverse effects such changes in locomotor activity and body temperature and seizure induction in mice. Our results show PNU-120596 at 8 mg/kg did not significantly alter nicotine-induced locomotor depression dose-response curve. In addition, administration of PNU-120596 (8 mg/kg) did not enhance nicotine-induced seizures, a response partially mediated by  $\alpha 7$  nAChRs (Damaj et al., 1999; Tritto et al., 2003; Stitzel et al., 1998; 2000). It is possible that mediation of seizure sensitivity is more closely related to a direct activation of  $\alpha 7$  nAChRs rather than an indirect one, as is the case with allosteric modulation. On its own, PNU-120596 (8 mg/kg) failed neither to either alters locomotor activity of mice nor to induce seizures or convulsions in these animals after i.p. injection.

Interestingly, PNU-120596 dose-dependently enhanced hypothermia induced by the lowest dose (0.5mg/kg) of nicotine. This enhancement disappeared at higher doses of the drug. The  $\alpha 7$  antagonist MLA blocked PNU-120596's enhancement of nicotine-induced hypothermia

suggesting the plausible involvement of  $\alpha 7$  nAChRs. In addition, in the presence of PNU-120596 mice were more sensitive to  $\alpha 7$  agonist PHA-543613's (8 mg/kg) effects on body temperature. On its own, PHA-543613 (8 mg/kg) did not induce a decrease of body temperature of the animals. These findings were surprising since nicotine-induced hypothermia is mediated by  $\beta 2$ -,  $\beta 4$ -,  $\alpha 4$ -,  $\alpha 5$ - but not  $\alpha 7$ -containing nAChRs (Tapper et al., 2007; Tritto et al. 2004; Sack et al., 2005; Jackson et al., 2010; Ortiz et al., 2012). The enhancement of nicotine's hypothermic effects seems to require positive allosteric modulation, since the  $\alpha 7$  direct agonist PHA-543613, failed to alter this nicotinic response. Potentiation of nicotine's effects might therefore be the result a higher calcium influx due to prolonging receptor activation by PNU-120596. Higher calcium influx has been demonstrated to induce hypothermia in murine hippocampal neurons (Warren et al., 2012). However, mechanisms behind PNU-120596 and nicotine interaction on body temperature are not clear. It has been suggested that nicotine produces hypothermia by interacting with presynaptic nicotinic receptors to release acetylcholine, which acts on muscarinic receptors (Gordon, 1994; Overstreet et al., 1998). Furthermore, dopaminergic and opiates mechanisms were reported to mediate nicotine's effects on the body temperature (Zarrindast and Tabatabai, 1992; Zarrindast and Abolfathi-Araghi, 1992; Sakoori and Murphy, 2009).

Lastly, we examined the anti-hyperalgesic and anti-allodynic effects of NS1738 and PNU-120596 in murine inflammatory and chronic neuropathic pain models after acute administration. Overall, our results showed that while NS1738 and PNU-120596 both attenuated hyperalgesia associated with inflammation, only PNU-120596 decreased hyperalgesia and allodynia in the chronic neuropathic pain model. Whereas PNU-120596 produced consistent effects in carrageenan and CCI models, there was a surprising disparity of the effects of NS1738

on hyperalgesia and edema. In addition, the effects of PNU-120596 were blocked by MLA, suggesting that  $\alpha 7$  nAChRs play a critical role in its action. Importantly, the antinociceptive effects of both drugs occurred at doses that had no effect on motor function and coordination in mice.

The difference in efficacy between PNU-120596 and NS1738 in the inflammatory, tonic and neuropathic pain models might be related to differences in  $\alpha 7$  nAChR regulation by these two drugs. Type I PAMs predominantly affect the apparent peak current with little effect on desensitization kinetics, whereas type II PAMs increase the apparent peak current and evoke a distinct weakly decaying current causing dramatic slowing of receptor desensitization (Hurst et al., 2005). Indeed, PNU-120596 was shown to activate  $\alpha 7$  nAChRs that would otherwise be desensitized (Papke et al., 2009). This difference in efficacy between NS1738 and PNU-120596 in our pain models was unexpected since both were previously reported to produce pro-cognitive effects in mice and rats (Hurst et al., 2005; Ng et al., 2007 and Thomsen et al., 2010).

PNU-120596's anti-inflammatory activity may also be explained by the inhibition of NF- $\kappa$ B, which induces macrophage activation and pro-inflammatory cytokine production (Lee et al., 1994; Baeuerle & Henkel, 1994). Support for this mechanism lays in the fact that NF- $\kappa$ B has been shown to reliably occur after carrageenan-induced paw edema and has been associated with nicotine's anti-inflammatory action (Bhattacharyya et al., 2008; Borthakur et al., 2007; Menegazzi et al., 2008; Min et al., 2009; Wang et al., 2004). It will be important in future studies to compare the effects of PNU-120596 and NS1738 in affecting the NF- $\kappa$ B pathway and pro-inflammatory cytokine production.

Moreover, PNU-120596 significantly reduced carrageenan-induced paw edema and thermal hyperalgesia after onset. This reversal of nociceptive and edematous responses has potential clinical significance, because anti-inflammatory drugs are typically given to patients after the onset of inflammation. Two explanations may account for PNU-120596's effects. First, PNU-120596, coupled to endogenous agonists, could be activating  $\alpha 7$  nAChRs, opening channels, and enhancing  $\text{Ca}^{2+}$  influx, therefore magnifying downstream effects. Although it has been reported that  $\text{Ca}^{2+}$  influx through a nAChR channel itself seems to contribute very little to the cytosolic concentration of calcium (Zhou and Neher, 1993), other studies report that  $\text{Ca}^{2+}$  permeability through  $\alpha 7$  nAChRs is important in cellular processes (Seguela et al., 1993). A recent study showed that  $\alpha 7$  nAChR-mediated calcium signaling and catecholamine release in bovine chromaffin cells requires PNU-120596 (del Barrio et al., 2010). There is also evidence for  $\alpha 7$  nAChRs-mediated noradrenaline release (Rowley and Flood, 2008) in the lumbar spinal cord as well as serotonin release (Cordero- Erasquin & Changeux, 2001). Second, PNU-120596, in the presence of endogenous ACh and choline, may activate  $\alpha 7$  nAChRs expressed in the nociceptive system and on macrophages, consequently inhibiting TNF- $\alpha$  synthesis (Bernik et al., 2002).

In addition to differences between the effects of NS1738 and PNU-120596 in the formalin and carrageenan inflammatory pain models, both drugs produced differential effects in the chronic neuropathic pain (CCI) model. While NS1738 (30mg/kg) was inactive in this model, PNU-120596 dose-dependently reduced thermal hyperalgesia and mechanical allodynia via  $\alpha 7$  nAChRs. Furthermore, PNU-120596 had no antinociceptive effect in contralateral paws (data not shown) or in sham-operated mice, indicating that it was antinociceptive under pain conditions, only. Considering NS1738's lack of effect in the CCI model, poor blood-brain

barrier permeability is improbable, since previous studies have shown that NS1738 was present at high enough concentrations in the brain after i.p. administration (Timmermann et al., 2007). Another possibility is receptor desensitization and  $\alpha 7$  nAChR regulation, as discussed earlier with the carrageenan model (Hurst et al., 2005; Papke et al., 2009). Moreover, inflammatory and neuropathic pain might have different pathomechanistic components (Walker et al., 1999), thus explaining NS1738's different effects in carrageenan-induced and CCI-induced pain models.

As  $\alpha 7$  PAMs presumably have the capability of enhancing  $\alpha 7$  agonist response (Gronlien et al., 2007; Hurst et al., 2005), we investigated the interaction between PNU-120596 and the selective  $\alpha 7$  agonist PHA-543613. As predicted, the combination of PNU-120596 and PHA-543613 produced enhanced anti-allodynic effects in the CCI model compared to the effects of each drug given alone. Therefore, these results suggest that PNU-120596 potentiates both endogenous cholinergic tone and the effect of exogenous  $\alpha 7$  agonist PHA-543613.

$\alpha 7$  nAChR-dependent neuroinflammatory processes may also be involved in the neuroprotective effects of  $\alpha 7$  PAMs in neuropathic pain. This hypothesis is supported by  $\alpha 7$  nAChR expression in anti-neuroinflammatory peripheral macrophages and murine microglia (Wang and Wang, 2003; Shytle et al., 2004; De Simone et al., 2005). A recent study showed that selective  $\alpha 7$  nAChR agonist PNU-282987 could reduce hyperalgesia, edema and macrophagic infiltration in the sciatic nerve.

The exact mechanisms involved in the antinociceptive effects of PNU-120596 in the chronic pain models are currently unknown. Previous work has related CCI-induced neuropathic pain and DRG  $\alpha 7$  nAChR up-regulation (Carnevale et al., 2007; Feuerbach et al., 2009; Fucile et al., 2005; Xiao et al., 2002). Thus, PNU-120596 could be enhancing endogenous cholinergic

tone through  $\alpha 7$  nAChRs in DRG neurons, reducing pain-related behaviors. PNU-120596 could also be acting through spinal  $\alpha 7$  nAChRs (Genzen & McGehee, 2005), though other reports claim that central  $\alpha 7$  nAChRs are not involved in thermal and mechanical nociceptive hypersensitivity in mice (Gao et al., 2010; Rashid et al., 2006). The discrepancies found in  $\alpha 7$  nAChR involvement in thermal nociceptive mediation may be due to the differential modulation of synapses and nociceptive properties, such as pain intensity, in various pathways (Martin et al., 1999; Millan, 2002; Yoshimura & Furue, 2006; Gao et al., 2010) and variation in methodology (ex. testing apparatus). Furthermore, PNU-120596 may indirectly, through excitatory neurons, or directly, through inhibitory neurons, modulate inhibitory neurotransmitter, such as serotonin, release. Cordero-Erausquin and Changeux (2001) have shown that ACh from excitatory neurons may tonically activate inhibitory interneuron nAChRs, possibly allowing PNU-120596 to enhance  $\alpha 7$  nAChR activation and indirectly activate inhibitory neurons. Aznar et al. (2005) even argues that  $\alpha 7$  nAChR activation directly effects inhibitory neuronal behavior; they suggest that  $\alpha 7$  nAChRs are present on inhibitory serotonin-releasing neurons that project to the hippocampus and the septum.

In summary, our results demonstrate for the first time that type I and II  $\alpha 7$  PAMs differentially potentiate agonist activation of  $\alpha 7$  nAChR in tonic, inflammatory and chronic neuropathic pain models *in vivo*. PNU-120596, but not NS1738, plays an important role in regulating pain-stimulated behavior in the formalin test and CCI model. The antinociceptive effects of PNU-120596 in the formalin test involve the activation of both central and peripheral  $\alpha 7$  nAChRs. Additionally, the interaction between PNU-120596 and choline has a synergistic antinociceptive effect in the formalin test. Lastly, we show that  $\alpha 7$  nAChR PAMs have anti-hyperalgesic and anti-allodynic properties in inflammatory and chronic neuropathic pain models.



Previous studies have compared behavioral responses after the administration of type I and II PAMs across different models of disorder, including neurodegeneration and cognitive deficits in learning, memory, attention; they have shown that these PAMs enhance agonist response and improve memory and cognition in rodents (Hogg & Bertrand, 2004; Thomsen et al., 2011; Hurst et al., 2005; Ng et al., 2007). Similarly, our study compared type I and II PAMs in inflammatory and chronic neuropathic pain models. Our results suggest that NS1738 and PNU-120596 differentially modulate endogenous cholinergic tone in these models, supporting findings by Thomsen et al. (2010) that NS1738 and PNU-120596 differently modulate agonist-induced upregulation of  $\alpha 7$  nAChR. Though PAMs are currently being developed for the treatment of cognitive deficits in patients with schizophrenia or Alzheimer's disease (Thomsen et al., 2010), we demonstrate that type II  $\alpha 7$  nAChR PAMs may be potential candidates for the management of inflammatory and chronic neuropathic pain.

## 5.2 Significance of Findings

Whereas efficacious treatment of chronic pain requires a multidisciplinary approach (Flor et al., 1992), pharmacotherapy plays a central role. A host of pharmacological treatments have been successful in managing pain to improve daily function of the individual, including opioids, anti-inflammatory steroids, NSAIDs, COX-2 inhibitors, antimigraines, anticonvulsants and antidepressants. While these pharmacological treatments are beneficial across a wide range of conditions, they are not uniformly effective, and undesired side effects frequently limit their use. Therefore, to date, pain, especially chronic pain, remains a prevalent therapeutic challenge. The increasingly understood role of the  $\alpha 7$  nAChR in pain and inflammation has recently established this subtype as a potential target.

Since PNU-120596 produced antinociceptive effects in the mouse models of inflammatory and chronic neuropathic pain, type II PAMs may be considered viable candidates for the development as analgesics. Though our results are not conclusive, genetic homology between mice and humans suggest that our findings may be translational. Genomic analyses between humans and mice have indicated an 85% homology between complete genomes (Rogers et al., 2007) and a 94% homology between the mouse and the human  $\alpha 7$  nAChR (BLAST search: [www.blast.ncbi.nlm.nih.gov.proxy.library.vcu.edu/Blast.cgi](http://www.blast.ncbi.nlm.nih.gov.proxy.library.vcu.edu/Blast.cgi)). Furthermore, *in vitro* functional studies with  $\alpha 7$  agonists and antagonists show that human and mouse  $\alpha 7$  nAChRs have similar pharmacological properties (Papke et al., 2010). Thus, the high structural and functional similarities homology of the  $\alpha 7$  nAChR in mice and humans supports the translational capacity of our studies and shows that the enhancement of  $\alpha 7$  nAChR activation by positive allosteric modulation is a promising approach for chronic pain treatment.

Classically, the aim of drug therapy has been to normalize pain sensitivity, such as that which contributes to behavioral conditions like allodynia and hyperalgesia, which have been induced in preclinical chronic pain models (Negus et al., 2006). Following this aim, our studies show that PNU-120596 produced anti-allodynic and anti-hyperalgesic effects. In addition to inducing these nociceptive behaviors, pain is known to suppress normally adaptive behaviors, such as walking, feeding, social interactions and affective behavior (Ostelo & de Vet, 2005).

Stevenson et al (2006) compared effects of morphine, a mu opioid analgesic, and haloperidol, an antipsychotic dopamine antagonist, in the suppression of feeding (a pain-suppressed behavior) and abdominal writhing (pain-stimulated behavior) in an acetic acid rodent model. Importantly, morphine prevented both of these aspects measured, whereas haloperidol was effective only in inhibiting acid-induced writhing. Therefore, pain-related behavior

suppression studies are needed to build a stronger bridge between clinical and preclinical measures of pain (Negus et al., 2006). The integration of findings from studies of PNU-120596's effectiveness in pain-stimulated behaviors and in pain-suppressed behaviors would institute a more relevant and translational profile of PNU-120596 as an analgesic.

There are two major mechanistic issues in regard to type II PAMs to be considered for their development as analgesic drugs. First, these PAMs dramatically prolong the duration of the agonist-evoked ions currents because they reduce receptor desensitization. Second, type II PAMs also promote the activation of previously desensitized receptors (Gronlien et al., 2007). The result is an increase in agonist-evoked intracellular calcium flux (Hurst et al., 2005), which raises the possibility of calcium-induced cytotoxicity in various cell types. Thus, several studies tried to address this issue. However, there is contradictory evidence about PNU-120596's *in vitro* cytotoxicity profile. For example, Ng et al (2007) showed that high concentrations of PNU-120596 are cytotoxic in  $\alpha 7$  nAChRs expressed in the SH-SY5Y- cell line. Likewise, Dinklo et al (2011) showed that PNU-120596 induced cellular toxicity in the GH4C1 cell line expressing the human  $\alpha 7$  nAChR. In contrast to these findings, Hu et al (2009) showed that PNU-120596 alone or in combination with agonist did not produce cytotoxic effects in PC12 cells and native primary cultures of rat cortical neurons. However, to date, there are no *in vivo* studies of neurotoxicity after chronic administration of these type II PAMs. The cause for this inconsistency still unclear, thus more studies is needed to evaluate the extent of this possible limitation.

Another possible limitation of our behavioral observations with PNU-120596, where we found that it enhanced nicotine-induced hypothermia. These interactions may have some implications for smokers and patients under nicotine replacement therapy. However, we are not

sure if these effects of PNU-120696 could be replicated by other PAM IIs or even PAM Is. Consequently, more studies are required to validate the use of PNU-120596 as an active therapeutic agent for chronic pain treatment.

### 5.3 Future Directions

While our results in mouse pain models are encouraging, more behavioral studies are needed. The full behavioral and pharmacological effects of PNU-120596 must be further studied. The reproducibility of these antinociceptive results in different animal species, such as rats or monkeys, would increase the translational value of our results. Additionally, a comprehensive study of PNU-120596's effects on the affective signs of pain could lead to a more translational evaluation of PNU-120596 as a potential therapy for chronic pain. For example, studies could be conducted to find the effect of PNU-120596 in models that measure pain-suppressed behaviors. Understanding the analgesic spectrum and profile of PNU-120596's effects in other types of pain such as cancer or burn pain would also be important to determine.

Key differences in efficacy between PNU-120596 and NS1738 in pain tests could reveal important molecular mechanistic features of these two types of PAMs. For example, previous studies have linked  $\alpha 7$  with adenosine monophosphate cyclic (cAMP) response element binding protein (pCREB) and protein kinase C (PKC), suggesting that PNU-120596's and NS1738's differences may involve signaling mechanisms such as pCREB and PKC. Additionally, PKC sustains nociceptive plasticity in spinal cord dorsal horn, which may play an important role in chronic pain.

Furthermore, a better understanding of the anti-inflammatory profile of PNU-120596's is needed. For example, a better grasp of PNU-120596's effect on mediators of inflammation such as TNF –  $\alpha$  or other cytokines would be an important step.

Finally, a more in-depth understanding of the interaction between PNU-120596 and nicotine and the mechanisms involved will be required. For example, it will be important to determine if other type II PAMs, that are structurally different from PNU-120596, would enhance nicotine-induced hypothermia.. The interaction of type I PAM with nicotine should also be explored. Lastly, it is important to determine if tolerance to the enhancement of nicotine-induced hypothermia develops after chronic exposure.

## References

- Abbott, F. V., Franklin, K. B., & Westbrook, R. F. (1995). The formalin test: scoring properties of the first and second phases of the pain response in rats. *Pain*, *60*(1), 91-102. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/7715946>
- Aceto, M.D., Bagley, R.S., Dewey, W.L., Fu, T.C., & Martin, B.R. (1986). The spinal cord as a major site for the antinociceptive action of nicotine in the rat. *Neuropharmacology*, *25*(9), 1031-6.
- Albuquerque, E.X., Santos, M.D., Alkondon, M., Pereira, E.F., & Maelicke, A. (2001). Modulation of nicotinic receptor activity in the central nervous system: a novel approach to the treatment of Alzheimer disease. *Alzheimer Disease and Associated Disorders*, *15 Suppl 1*, S19-25.
- Aley, K. O., Martin, a, McMahon, T., Mok, J., Levine, J. D., & Messing, R. O. (2001). Nociceptor sensitization by extracellular signal-regulated kinases. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *21*(17), 6933-9. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/11517280>
- Alkondon, M, Pereira, E. F., Cortes, W. S., Maelicke, a, & Albuquerque, E. X. (1997). Choline is a selective agonist of alpha7 nicotinic acetylcholine receptors in the rat brain neurons. *The European journal of neuroscience*, *9*(12), 2734-42. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/9517478>
- Alkondon, Manickavasagom, & Albuquerque, E. X. (2006). Subtype-Specific Inhibition of Nicotinic Acetylcholine Receptors by Choline : A Regulatory Pathway, *318*(1), 268-275. doi:10.1124/jpet.106.103135.asterisk
- Allen, D. ., & Lockman, P. . (2003). The blood-brain barrier choline transporter as a brain drug delivery vector. *Life Sciences*, *73*(13), 1609-1615. doi:10.1016/S0024-3205(03)00504-6
- Alter, B. J., Zhao, C., Karim, F., Landreth, G. E., & Gereau, R. W. (2010). Genetic targeting of ERK1 suggests a predominant role for ERK2 in murine pain models. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *30*(34), 11537-47. doi:10.1523/JNEUROSCI.6103-09.2010
- Andurkar, S. V., & Gulati, A. (2011). Assessment of the analgesic effect of centhaquin in mouse tail flick and hot-plate tests. *Pharmacology*, *88*(5-6), 233-41. doi:10.1159/000331880
- Arner, S., & Meyerson, B.A. (1988). Lack of analgesic effect of opioids on neuropathic and idiopathic forms of pain. *Pain*, *33*(1), 11-23.
- Aslan, E., Kocaeli, H., Bekar, A., Tolunay, S., & Ulus, I. H. (2011). CDP-choline and its endogenous metabolites, cytidine and choline, promote the nerve regeneration and

- improve the functional recovery of injured rat sciatic nerves. *Neurological research*, 33(7), 766-73. doi:10.1179/1743132811Y.0000000004
- Attal, N., & Bouhassira, D. (1999) Mechanisms of pain in peripheral neuropathy. *Acta Neurologica Scandinavica Supplementum* 173:48-52.
- Aznar, S., Kostova, V., Christiansen, S. H., & Knudsen, G. M. (2005). Alpha 7 nicotinic receptor subunit is present on serotonin neurons projecting to hippocampus and septum. *Synapse (New York, N.Y.)*, 55(3), 196-200. doi:10.1002/syn.20108
- Baeuerle, P. A., & Henkel, T. (1994). Function and activation of NF-kB in the immune system. *Annual Review Immunology*, 12, 141-179.
- Bannon, A. W., Gunther, K. L., & Decker, W. (1995). Is Epibatidine Really Analgesic ? Dissociation of the Activity , Temperature , and Analgesic Effects of (+)-Epibatidine, 51(4), 693-698.
- Barron, S. C., Mclaughlin, J. T., See, J. A., Richards, V. L., & Rosenberg, R. L. (2009). An Allosteric Modulator of  $\alpha$  7 Nicotinic Receptors , N- ( 5- ( PNU-120596 ), Causes Conformational Changes in the Extracellular Ligand Binding Domain Similar to Those Caused by Acetylcholine  $\square$ . *Molecular Pharmacology*. doi:10.1124/mol.109.056226.
- Baumann, H., & Gauldie, J. (1994). The acute phase response. *Immunol Today* 15, 74-80.
- Bencherif, M., Lippiello, P. M., Lucas, R., & Marrero, M. B. (2011). Alpha7 nicotinic receptors as novel therapeutic targets for inflammation-based diseases. *Cellular and molecular life sciences : CMLS*, 68(6), 931-49. doi:10.1007/s00018-010-0525-1
- Bendszus, M., & Stoll, G. (2003). Caught in the act: in vivo mapping of macrophage infiltration in nerve injury by magnetic resonance imaging. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 23(34), 10892-6. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/14645484>
- Bernik, T. R., Friedman, S. G., Ochani, M., DiRaimo, R., Ulloa, L., Yang, H., Sudan, S., et al. (2002). Pharmacological stimulation of the cholinergic antiinflammatory pathway. *The Journal of experimental medicine*, 195(6), 781-8. Retrieved from <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2193742&tool=pmcentrez&rendertype=abstract>
- Bertrand, D., Bertrand, S., & Ballivet M. (1992). Pharmacological properties of the homomeric alpha 7 receptor. *Neuroscience Letters*, 146(1), 87-90.
- Bertrand, D., & Gopalakrishnan, M. (2007). Allosteric modulation of nicotinic acetylcholine receptors. *Biochemical pharmacology*, 74(8), 1155-63. doi:10.1016/j.bcp.2007.07.011

- Bhattacharyya, S., Dudeja, P.K., & Tobacman, J.K. (2008). Carrageenan-induced NFkappaB activation depends on distinct pathways mediated by reactive oxygen species and Hsp27 or by Bcl10. *Biochimica et Biophysica Acta*, 1780(7-8), 973-982.
- Bilbe, G., McAllister, K., & Hoyer, D. (2008). The selective nicotinic acetylcholine receptor alpha7 agonist JN403 is active in animal models of cognition, sensory gating, epilepsy and pain. *Neuropharmacology*, 56(1):254-263.
- Bitner, R Scott, Bunnelle, W. H., Decker, M. W., Drescher, K. U., Kohlhaas, K. L., Markosyan, S., Marsh, K. C., et al. (2010). In Vivo Pharmacological Characterization of a Novel Selective  $\alpha 7$  Neuronal Nicotinic Acetylcholine Receptor Agonist ABT- 107 : Preclinical Considerations in Alzheimer ' s Disease, 334(3), 875-886. doi:10.1124/jpet.110.167213.neuroscience
- Bitner, R S, Nikkel, a L., Curzon, P., Arneric, S. P., Bannon, a W., & Decker, M. W. (1998). Role of the nucleus raphe magnus in antinociception produced by ABT-594: immediate early gene responses possibly linked to neuronal nicotinic acetylcholine receptors on serotonergic neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 18(14), 5426-32. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/9651224>
- Blackburn-Munro, G. (2004). Pain-like behaviours in animals - how human are they? *Trends in pharmacological sciences*, 25(6), 299-305. doi:10.1016/j.tips.2004.04.008
- Boorman, J. P., Beato, M., Groot-Kormelink, P. J., Broadbent, S. D., & Sivilotti, L. G. (2003). The effects of beta3 subunit incorporation on the pharmacology and single channel properties of oocyte-expressed human alpha3beta4 neuronal nicotinic receptors. *The Journal of biological chemistry*, 278(45), 44033-40. doi:10.1074/jbc.M211719200
- Borthakur, A., Bhattacharyya, S., Dudeja, P.K., & Tobacman, J.K. (2007). Carrageenan induces interleukin-8 production through distinct Bcl10 pathway in normal human colonic epithelial cells. *American Journal of Physiology. Gastrointestinal and Liver Physiology*. 292(3), G829-838.
- Bridges, D., Thompson, S. W., & Rice, a S. (2001). Mechanisms of neuropathic pain. *British journal of anaesthesia*, 87(1), 12-26. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/11460801>
- Brunzell, D. H., & McIntosh, J. M. (2012). Alpha7 nicotinic acetylcholine receptors modulate motivation to self-administer nicotine: implications for smoking and schizophrenia. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, 37(5), 1134-43. Nature Publishing Group. doi:10.1038/npp.2011.299



- Buccafusco, J. J., Beach, J. W., & Terry, A. V. (2009). Desensitization of Nicotinic Acetylcholine Receptors as a Strategy for Drug Development. *Discovery*, 328(2), 364-370. doi:10.1124/jpet.108.145292.nicotine
- Buccafusco, J., Letchworth, S., Bencherif, M., & Lippiello, P. (2005). Long-lasting cognitive improvement with nicotinic receptor agonists: mechanisms of pharmacokinetic–pharmacodynamic discordance. *Trends in Pharmacological Sciences*, 26(7), 352-360. doi:10.1016/j.tips.2005.05.007
- Calmels, P., Mick, G., Perrouin-Verbe, B., & Ventura, M. (2009). Neuropathic pain in spinal cord injury: identification, classification, evaluation. *Annals of physical and rehabilitation medicine*, 52(2), 83-102. doi:10.1016/j.rehab.2008.12.012
- Carnevale, D., De Simone, R., & Minghetti, I. (2007). Microglia-neuron interaction in inflammatory and degenerative diseases: role of cholinergic and noradrenergic systems. *CNS and Neurological Disorders Drug Targets* 6(6), 388-397.
- Castro, N. G., & Albuquerque, E. X. (1995). alpha-Bungarotoxin-sensitive hippocampal nicotinic receptor channel has a high calcium permeability. *Biophysical journal*, 68(2), 516-24. doi:10.1016/S0006-3495(95)80213-4
- Chang, C., & Shyu, B. C. (2001). A fMRI study of brain activations during non-noxious and noxious electrical stimulation of the sciatic nerve of rats. *Brain research*, 897(1-2), 71-81. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/11282360>
- Chaplan, S.R., Bach, F.W., Pogrel, J.W., Chung, J.M., & Yaksh, T.L. (1994). Quantitative assessment of tactile allodynia in the rat paw. *Journal of Neuroscience Methods*, 53(1), 55-63.
- Christensen, D. Z., Mikkelsen, J. D., Hansen, H. H., & Thomsen, M. S. (2010). Repeated administration of alpha7 nicotinic acetylcholine receptor (nAChR) agonists, but not positive allosteric modulators, increases alpha7 nAChR levels in the brain. *Journal of neurochemistry*, 114(4), 1205-16. doi:10.1111/j.1471-4159.2010.06845.x
- Christopoulos, A. (2002). Allosteric binding sites on cell-surface receptors: novel targets for drug discovery. *Nature reviews. Drug discovery*, 1(3), 198-210. doi:10.1038/nrd746
- Collins, T., Young, G. T., & Millar, N. S. (2011). Competitive binding at a nicotinic receptor transmembrane site of two  $\alpha 7$ -selective positive allosteric modulators with differing effects on agonist-evoked desensitization. *Neuropharmacology*, 61(8), 1306-1313. Elsevier Ltd. doi:10.1016/j.neuropharm.2011.07.035
- Conejero-Goldberg, C., Davies, P., & Ulloa, L. (2008). Alpha7 nicotinic acetylcholine receptor: A link between inflammation and neurodegeneration, 32(4), 693-706. doi:10.1016/j.neubiorev.2007.10.007.Alpha7

- Cordero-Erausquin, M., & Changeux, J. P. (2001). Tonic nicotinic modulation of serotonergic transmission in the spinal cord. *Proceedings of the National Academy of Sciences of the United States of America*, 98(5), 2803-7. doi:10.1073/pnas.041600698
- Cordero-Erausquin, M., Marubio, L. M., Klink, R., & Changeux, J. P. (2000). Nicotinic receptor function: new perspectives from knockout mice. *Trends in pharmacological sciences*, 21(6), 211-7. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/10838608>
- Cordero-Erausquin, Matilde, Pons, S., Faure, P., & Changeux, J.-P. (2004). Nicotine differentially activates inhibitory and excitatory neurons in the dorsal spinal cord. *Pain*, 109(3), 308-18. doi:10.1016/j.pain.2004.01.034
- D'Amour, F.E., & Smith, D.L. (1941). A method for determining loss of pain sensation. *Journal of Pharmacology and Experimental Therapeutics*, 72, 74-79.
- Damaj, M. I., Fei-Yin, M., Dukat, M., Glassco, W., Glennon, R. a., & Martin, B. R. (1998). Antinociceptive responses to nicotinic acetylcholine receptor ligands after systemic and intrathecal administration in mice. *The Journal of pharmacology and experimental therapeutics*, 284(3), 1058-65. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/9495867>
- Damaj, M. I., Glassco, W., Dukat, M., & Martin, B. R. (1999). Pharmacological characterization of nicotine-induced seizures in mice. *The Journal of pharmacology and experimental therapeutics*, 291(3), 1284-91. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/10565853>
- Damaj, M. I., Meyer, E. M., & Martin, B. R. (2000). The antinociceptive effects of alpha7 nicotinic agonists in an acute pain model. *Neuropharmacology*, 39(13), 2785-91. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/11044748>
- Dani, J.A. (2001). Overview of nicotinic receptors and their roles in the central nervous system. D-aspartic acid or beta-amyloid peptide occur through independent mechanisms distinguished by pro-inflammatory cytokines. *Journal of Neurochemistry*, 87(5), 1125-1136.
- Davey, A. E., Leach, K., Valant, C., Conigrave, A. D., Sexton, P. M., & Christopoulos, A. (2012). Positive and negative allosteric modulators promote biased signaling at the calcium-sensing receptor. *Endocrinology*, 153(3), 1232-41. doi:10.1210/en.2011-1426
- Davidson, E. M., & Carlton, S. M. (1998). Intraplantar injection of dextrorphan, ketamine or memantine attenuates formalin-induced behaviors. *Brain research*, 785(1), 136-42. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/9526066>
- De Simone, R., Ajmone-Cat, M. A., Carnevale, D., & Minghetti, L. (2005). Activation of alpha7 nicotinic acetylcholine receptor by nicotine selectively up-regulates cyclooxygenase-2

- and prostaglandin E2 in rat microglial cultures. *Journal of neuroinflammation*, 2(1), 4. doi:10.1186/1742-2094-2-4
- Decker, M. W., Meyer, M. D., & Sullivan, J. P. (2001). The therapeutic potential of nicotinic acetylcholine receptor agonists for pain control. *Expert opinion on investigational drugs*, 10(10), 1819-30. doi:10.1517/13543784.10.10.1819
- Decker, M.W., Rueter, L.E., & Bitner, R.S. (2004). Nicotinic acetylcholine receptor agonists: a potential new class of analgesics. *Current topics in medicinal chemistry*, 4(3), 369-84.
- Detloff, M. R., Clark, L. M., Hutchinson, K. J., Kloos, A. D., Fisher, L. C., & Basso, D. M. (2010). Validity of acute and chronic tactile sensory testing after spinal cord injury in rats. *Experimental neurology*, 225(2), 366-76. Elsevier B.V. doi:10.1016/j.expneurol.2010.07.009
- Dewey, W. L., Harris, L. S., Howes, J. F., & Nuite, J. A. (1970). The effect of various neurohumoral modulators on the activity of morphine and the narcotic antagonists in the tail-flick and phenylquinone tests. *The Journal of pharmacology and experimental therapeutics*, 175(2), 435-442.
- Ding, M., Ghanekar, S., Elmore, C. S., Zysk, J. R., Werkheiser, J. L., Lee, C.-M., Liu, J., et al. (2012). [<sup>3</sup>H]Chiba-1001(methyl-SSR180711) has low in vitro binding affinity and poor in vivo selectivity to nicotinic alpha-7 receptor in rodent brain. *Synapse (New York, N.Y.)*, 66(4), 315-22. doi:10.1002/syn.21513
- Dinklo, T., Shaban, H., Thuring, J. W., Lavreysen, H., Stevens, K. E., Zheng, L., Mackie, C., et al. (2011). 4- (4-pyridinyl) -5-thiazolemethanol ( JNJ-1930942 ), a Novel Positive, 560-574. doi:10.1124/jpet.110.173245.
- Dixon, W. (1965). The up-and-down method for small samples. *Journal of American Statisticians Association*, 60, 967-978.
- Doble, A. (1999). New insights into the mechanism of action of hypnotics. *Journal of Psychopharmacology*, 13(4 Suppl 1), S11-20.
- Donnelly-Roberts, D., Malysz, J., Faghih, R., Gronlien, H., Haakerud, M., Thorin-Hagne, K., Ween, H., Gopalakrishnan, S.M., Li, J., Anderson, D.J., Kohlhaas, K., Namovic, M., Radek, R., Robb, H., Briggs, C.A., Bitner, R.S., Bunnelle, & Gopalakrishnan M (2009) Profile of A-716096, a novel thiazolylidine positive allosteric modulator of the  $\alpha 7$  nicotinic acetylcholine receptor. *Biochemical Pharmacology*, 78, 912.
- Dray, A., & Bevan, S. (1993). Inflammation and hyperalgesia: highlighting the team effort. *Trends in Pharmacological Sciences*, 14(8), 287-290.
- Dubuisson, D., & Dennis, S.G. (1977). The formalin test: a quantitative study of the

- analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. *Pain*, 4(2), 161-74.
- Duncia, J. V., Santella, J. B., Higley, C. a, Pitts, W. J., Wityak, J., Fietze, W. E., Rankin, F. W., et al. (1998). MEK inhibitors: the chemistry and biological activity of U0126, its analogs, and cyclization products. *Bioorganic & medicinal chemistry letters*, 8(20), 2839-44. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/9873633>
- Dziewczapolski, G., Glogowski, C. M., Masliah, E., & Heinemann, S. F. (2009). Deletion of the alpha 7 nicotinic acetylcholine receptor gene improves cognitive deficits and synaptic pathology in a mouse model of Alzheimer's disease. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 29(27), 8805-15. doi:10.1523/JNEUROSCI.6159-08.2009
- El Kouhen, R., Hu, M., Anderson, D. J., Li, J., & Gopalakrishnan, M. (2009). Pharmacology of alpha7 nicotinic acetylcholine receptor mediated extracellular signal-regulated kinase signalling in PC12 cells. *British journal of pharmacology*, 156(4), 638-48. doi:10.1111/j.1476-5381.2008.00069.x
- Faghih, R., Gfesser, G. A., & Gopalakrishnan, M. (2007). Advances in Discovery of Novel Positive Allosteric Modulators of the alpha7 Nicotinic Acetylcholine Receptor. *Recent Patents on CNS Drug Discovery*, 2, 99-106.
- Faghih, R., Gopalakrishnan, M., & Briggs, C. a. (2008). Allosteric modulators of the alpha7 nicotinic acetylcholine receptor. *Journal of medicinal chemistry*, 51(4), 701-12. doi:10.1021/jm070256g
- Feghali, C.A., & Wright, T.M. (1997). Cytokines in acute and chronic inflammation. *Frontiers in Bioscience*, 2, d12-26.
- Feuerbach, D., Lingenhoehl, K., Olpe, H.-R., Vassout, A., Gentsch, C., Chaperon, F., Nozulak, J., et al. (2009). The selective nicotinic acetylcholine receptor alpha7 agonist JN403 is active in animal models of cognition, sensory gating, epilepsy and pain. *Neuropharmacology*, 56(1), 254-63. Elsevier Ltd. doi:10.1016/j.neuropharm.2008.08.025
- Flor, H., Fydrich, T., & Turk, D.C. (1992). Efficacy of multidisciplinary pain treatment centers: a meta-analytic review. *Pain*, 49(2), 221-230.
- Flower, R. J. (2003). Studies on the mechanism of action of anti-inflammatory drugs. *Thrombosis Research*, 110(5-6), 259-263. doi:10.1016/S0049-3848(03)00410-9
- Fucile, S., Sucapane, A., & Eusebi, F. (2005). Ca<sup>2+</sup> permeability of nicotinic acetylcholine receptors from rat dorsal root ganglion neurones. *The Journal of Physiology*, 565(Pt 1), 219-228.

- Fujii, T., Takada-Takatori, Y., & Kawashima, K. (2008). Basic and Clinical Aspects of Non-neuronal Acetylcholine: Expression of an Independent, Non-neuronal Cholinergic System in Lymphocytes and Its Clinical Significance in Immunotherapy. *Journal of Pharmacological Sciences*, 106(2), 186-192. doi:10.1254/jphs.FM0070109
- Fujiwara, N., & Kobayashi, K. (2005). Macrophages in inflammation. *Current. Drug Targets. Inflammation and Allergy*, 4(3), 281-6.
- Gahring, L. C., Meyer, E. L., & Rogers, S. W. (2003). Nicotine-induced neuroprotection against N-methyl-d-aspartic acid or  $\beta$ -amyloid peptide occur through independent mechanisms distinguished by pro-inflammatory cytokines. *Journal of Neurochemistry*, 87(5), 1125-1136. doi:10.1046/j.1471-4159.2003.02074.x
- Gao, B., Hierl, M., Clarkin, K., Juan, T., Nguyen, H., Valk, M. V. D., Deng, H., et al. (2010). Pharmacological effects of nonselective and subtype-selective nicotinic acetylcholine receptor agonists in animal models of persistent pain. *Pain*, 149(1), 33-49. International Association for the Study of Pain. doi:10.1016/j.pain.2010.01.007
- Genzen, J. R., & McGehee, D.S. (2003). Nicotinic modulation of GABAergic synaptic transmission in the spinal cord dorsal horn. *Brain Research*, 1031(2), 229-237.
- Gessner, P. K. (1995). Isobolographic analysis of interactions : applications and utility an update on. *Toxicology*, 105, 161-179.
- Gillberg, P.G., & Aquilonius, S.M. (1985). Cholinergic, opioid and glycine receptor binding sites localized in human spinal cord by in vitro autoradiography. Changes in amyotrophic lateral sclerosis. *Acta Neurologica Scandinavica* 72(3), 299-306.
- Gillberg, P.G., Hartvig, P., Gordh, T., Sottile, A., Jansson, I., Archer, T., & Post, C. (1990). Behavioral effects after intrathecal administration of cholinergic receptor agonists in the rat. *Psychopharmacology (Berl)*, 100(4), 464-469.
- Girod, R., Crabtree, G., Ernstrom, G., Ramirez-Latorre, J., McGehee, D., Turner, J., & Role, L. (1999). Heteromeric Complexes of  $\alpha 5$  and / or  $\alpha 7$  Subunits. *Annals of the New York Academy of Sciences*, 868, 578-590.
- González-Rubio, J.M., Rojo, J., Tapia, L., Maneu, V., Mulet, J., Valor, L.M., Criado, M., Sala, F., García, A.G., & Gandía, L. (2006). Activation and blockade by choline of bovine  $\alpha 7$  and  $\alpha 3\beta 4$  nicotinic receptors expressed in oocytes. *European Journal of Pharmacology*, 535(1-3), 53-60.
- Gordon, C. J. (1994). 24-hour control of body temperature in the rat: II. Diisopropyl fluorophosphate-induced hypothermia and hyperthermia. *Pharmacology, biochemistry, and behavior*, 49(3), 747-54. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/7862732>

- Gotti, C, & Clementi, F. (2004). Neuronal nicotinic receptors: from structure to pathology. *Progress in neurobiology*, 74(6), 363-96. doi:10.1016/j.pneurobio.2004.09.006
- Gotti, Cecilia, Zoli, M., & Clementi, F. (2006). Brain nicotinic acetylcholine receptors: native subtypes and their relevance. *Trends in pharmacological sciences*, 27(9), 482-91. doi:10.1016/j.tips.2006.07.004
- Grønlien, J. H., Håkerud, M., Ween, H., Thorin-hagene, K., Briggs, C. A., Gopalakrishnan, M., & Malysz, J. (2007). Distinct Profiles of  $\alpha 7$  nAChR Positive Allosteric Modulation Revealed by Structurally Diverse Chemotypes. *Molecular Pharmacology*, 72(3), 715-724. doi:10.1124/mol.107.035410.tamers
- Gurun, M. S., Parker, R., Eisenach, J. C., & Vincler, M. (2009). The effect of peripherally administered CDP-choline in an acute inflammatory pain model: the role of alpha7 nicotinic acetylcholine receptor. *Anesthesia and analgesia*, 108(5), 1680-7. doi:10.1213/ane.0b013e31819dcd08
- Gusev, A. G., & Uteshev, V. V. (2010). Physiological Concentrations of Choline Activate Native  $\alpha 7$ -Containing Nicotinic Acetylcholine Receptors in the Presence. *Pharmacology*, 332(2), 588-598. doi:10.1124/jpet.109.162099.2004
- Hama, a, & Menzaghi, F. (2001). Antagonist of nicotinic acetylcholine receptors (nAChR) enhances formalin-induced nociception in rats: tonic role of nAChRs in the control of pain following injury. *Brain research*, 888(1), 102-106. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/11146056>
- Hamurtekin, E., & Gurun, M. S. (2006). The antinociceptive effects of centrally administered CDP-choline on acute pain models in rats: the involvement of cholinergic system. *Brain research*, 1117(1), 92-100. doi:10.1016/j.brainres.2006.07.118
- Han, K.-J., Choi, S.-S., Lee, J.-Y., Lee, H.-K., Shim, E.-J., Kwon, M. S., Seo, Y.-J., et al. (2005). Antinociceptive effect of nicotine in various pain models in the mouse. *Archives of pharmacal research*, 28(2), 209-15. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/15789753>
- Harden, R. N. (2005). Chronic neuropathic pain. Mechanisms, diagnosis, and treatment. *The neurologist*, 11(2), 111-22. doi:10.1097/01.nrl.0000155180.60057.8e
- Hardt, J., Jacobsen, C., Goldberg, J., Nickel, R., & Buchwald, D. (2008). Prevalence of chronic pain in a representative sample in the United States. *Pain medicine (Malden, Mass.)*, 9(7), 803-12. doi:10.1111/j.1526-4637.2008.00425.x
- Haydar, S.N., & Dunlop J (2010). Neuronal nicotinic acetylcholine receptors - targets for the development of drugs to treat cognitive impairment associated with schizophrenia and Alzheimer's disease. *Current Topics in Medicinal Chemistry*, 10(2), 144-152.



- Hejmadi, M.V., Dajas-Bailador, F., Barns, S.M., Jones, B., & Wonnacott, S. (2003). Neuroprotection by nicotine against hypoxia-induced apoptosis in cortical cultures involves activation of multiple nicotinic acetylcholine receptor subtypes. *Molecular and Cellular Neurosciences*, 24(3), 779-786.
- Hevers, W., & Lüddens, H. (1998). The diversity of GABAA receptors. Pharmacological and electrophysiological properties of GABAA channel subtypes. *Molecular neurobiology*, 18(1), 35-86. doi:10.1007/BF02741459
- Hogg, R.C., & Bertrand, D. (2004). Nicotinic acetylcholine receptors as drug targets. *CNS & Neurological Disorders*, 3(2), 123-130.
- Hood, D. D., Mallak, K. a, James, R. L., Tuttle, R., & Eisenach, J. C. (1997). Enhancement of analgesia from systemic opioid in humans by spinal cholinesterase inhibition. *The Journal of pharmacology and experimental therapeutics*, 282(1), 86-92. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/9223543>
- Hu, M., Gopalakrishnan, M., & Li, J. (2009). Positive allosteric modulation of  $\alpha 7$  neuronal nicotinic acetylcholine receptors: lack of cytotoxicity in PC12 cells and rat primary cortical neurons. *British Journal of Pharmacology*, 158(8), 1857-1864. doi:10.1111/j.1476-5381.2009.00474.x
- Huang, G.-J., Pan, C.-H., & Wu, C.-H. (2012). Sclareol exhibits anti-inflammatory activity in both lipopolysaccharide-stimulated macrophages and the  $\lambda$ -carrageenan-induced paw edema model. *Journal of natural products*, 75(1), 54-9. doi:10.1021/np200512a
- Hunskar, S., & Hole, K. (1987). The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. *Pain*, 30(1), 103-114.
- Hurst, R. S., Hajo, M., Raggenbass, M., Wall, T. M., Higdon, N. R., Lawson, J. A., Rutherford-root, K. L., et al. (2005). A Novel Positive Allosteric Modulator of the  $\alpha 7$  Neuronal Nicotinic Acetylcholine Receptor : In Vitro and In Vivo Characterization, 25(17), 4396-4405. doi:10.1523/JNEUROSCI.5269-04.2005
- Jackson, K. J., Marks, M. J., Vann, R. E., Chen, X., Gamage, T. F., Warner, J. A., & Damaj, M. I. (2010). Role of  $\alpha 5$  Nicotinic Acetylcholine Receptors in Pharmacological and Behavioral Effects of Nicotine in Mice. doi:10.1124/jpet.110.165738.
- Jackson, K. J., Walters, C. L., & Damaj, M. I. (2009).  $\alpha 2$  Subunit-Containing Nicotinic Receptors Mediate Acute Nicotine-Induced Activation of Calcium / Calmodulin-Dependent Protein Kinase II-Dependent Pathways in Vivo, 330(2), 541-549. doi:10.1124/jpet.109.153171.tivates
- Kalappa, B. I., Gusev, A. G., & Uteshev, V. V. (2010a). Activation of functional  $\alpha 7$ -containing nAChRs in hippocampal CA1 pyramidal neurons by physiological levels of choline in the presence of PNU-120596. *PloS one*, 5(11), e13964. doi:10.1371/journal.pone.0013964

- Kalappa, B. I., Gusev, A. G., & Uteshev, V. V. (2010b). Activation of functional  $\alpha 7$ -containing nAChRs in hippocampal CA1 pyramidal neurons by physiological levels of choline in the presence of PNU-120596. *PloS one*, 5(11), e13964. doi:10.1371/journal.pone.0013964
- Kanaan, S. a, Saadé, N. E., Haddad, J. J., Abdelnoor, a M., Atweh, S. F., Jabbur, S. J., & Safieh-Garabedian, B. (1996). Endotoxin-induced local inflammation and hyperalgesia in rats and mice: a new model for inflammatory pain. *Pain*, 66(2-3), 373-9. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/8880861>
- Kaneko, S., Maeda, T., Kume, T., Kochiyama, H., Akaike, a, Shimohama, S., & Kimura, J. (1997). Nicotine protects cultured cortical neurons against glutamate-induced cytotoxicity via alpha7-neuronal receptors and neuronal CNS receptors. *Brain research*, 765(1), 135-40. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/9310404>
- Karlsten, R., & Gordh, T. (1997). How do drugs relieve neuropathic pain? *Drugs Aging*, 11, 398-412.
- Kawashima, K., & Fujii, T. (2003). The lymphocytic cholinergic system and its contribution to the regulation of immune activity. *Life Sciences*, 74(6), 675-696. doi:10.1016/j.lfs.2003.09.037
- Kayser, V., & Guilbaud, G. (1987). Local and remote modifications of nociceptive sensitivity during carrageenan-induced inflammation in the rat. *Pain*, 28(1), 99-107.
- Kesingland, a C., Gentry, C. T., Panesar, M. S., Bowes, M. a, Vernier, J. M., Cube, R., Walker, K., et al. (2000). Analgesic profile of the nicotinic acetylcholine receptor agonists, (+)-epibatidine and ABT-594 in models of persistent inflammatory and neuropathic pain. *Pain*, 86(1-2), 113-8. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/10779668>
- Khan, I. M., Stanislaus, S., Zhang, L., Taylor, P., & Yaksh, T. L. (2001). A-85380 and epibatidine each interact with disparate spinal nicotinic receptor subtypes to achieve analgesia and nociception. *The Journal of pharmacology and experimental therapeutics*, 297(1), 230-9. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/11259549>
- Khan, I. M., Taylor, P., & Yaksh, T. L. (1994). Cardiovascular and behavioral responses to nicotinic agents administered intrathecally. *The Journal of pharmacology and experimental therapeutics*, 270(1), 150-8. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/8035311>
- Khan, I. M., Yaksh, T. L., & Taylor, P. (1997). Epibatidine binding sites and activity in the spinal cord. *Brain research*, 753(2), 269-82. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/9125412>
- Khan, I., Osaka, H., Stanislaus, S., Calvo, R. M., Deerinck, T., Yaksh, T. L., & Taylor, P. (2003). Nicotinic acetylcholine receptor distribution in relation to spinal neurotransmission pathways. *The Journal of comparative neurology*, 467(1), 44-59. doi:10.1002/cne.10913



- Kiyosawa, a, Katsurabayashi, S., Akaike, N., Pang, Z. P., & Akaike, N. (2001). Nicotine facilitates glycine release in the rat spinal dorsal horn. *The Journal of physiology*, 536(Pt 1), 101-10. Retrieved from <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2278850&tool=pmcentrez&rendertype=abstract>
- Kocher, L., Anton, F., Reeh, P.W., & Handwerker, H.O. (1987). The effect of carrageenan-induced inflammation on the sensitivity of unmyelinated skin nociceptors in the rat. *Pain* 29(3), 363-373.
- Kominato, Y., Tachibana, T., Dai, Y., Tsujino, H., Maruo, S., & Noguchi, K. (2003). Changes in phosphorylation of ERK and Fos expression in dorsal horn neurons following noxious stimulation in a rat model of neuritis of the nerve root. *Brain research*, 967(1-2), 89-97. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/12650969>
- Kurzen, H., Wessler, I., Kirkpatrick, C. J., Kawashima, K., & Grando, S. a. (2007). The non-neuronal cholinergic system of human skin. *Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et métabolisme*, 39(2), 125-35. doi:10.1055/s-2007-961816
- Lambert, G. a, Mallos, G., & Zagami, A. S. (2009). Von Frey's hairs--a review of their technology and use--a novel automated von Frey device for improved testing for hyperalgesia. *Journal of neuroscience methods*, 177(2), 420-6. doi:10.1016/j.jneumeth.2008.10.033
- Le Bars, D., Gozariu, M., & Cadden, S. W. (2001). Animal models of nociception. *Pharmacological reviews*, 53(4), 597-652. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/21341540>
- Lee, C.-H., Zhu, C., Malysz, J., Campbell, T., Shaughnessy, T., Honore, P., Polakowski, J., et al. (2011).  $\alpha 4\beta 2$  neuronal nicotinic receptor positive allosteric modulation: an approach for improving the therapeutic index of  $\alpha 4\beta 2$  nAChR agonists in pain. *Biochemical pharmacology*, 82(8), 959-66. doi:10.1016/j.bcp.2011.06.044
- Lee, J. C., Laydon, J. T., McDonnell, P. C., Gallagher, T. F., Kumar, S., Green, D., McNulty, D., et al. (1994). A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature*, 372, 739-746.
- Li, X., Pan, H.L., Xu, Z., Tobin, J.R. (2000). Nicotinic cholinergic receptors mediates norepinephrine release in rat spinal cord: an in vivo microdialysis study. *Journal of Neuroscience*, 26, 356-359.
- Li, X., Rainnie, D. G., Mccarley, R. W., & Greene, R. W. (1998). Monoaminergic Transmission, 18(5), 1904-1912.

- Lloyd, G. K., & Williams, M. (2000). Neuronal nicotinic acetylcholine receptors as novel drug targets. *The Journal of pharmacology and experimental therapeutics*, 292(2), 461-7. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/10640281>
- López-hernández, G. Y., Thinschmidt, J. S., Morain, P., Trocme-, C., Kem, W. R., Soti, F., & Papke, R. L. (2010). NIH Public Access, 56(4), 821-830. doi:10.1016/j.neuropharm.2009.01.011.Positive
- Maelicke, A., & Albuquerque, E. X. (2000). Allosteric modulation of nicotinic acetylcholine receptors as a treatment strategy for Alzheimer ' s disease, *c*, 165-170.
- Mannion, R. J., & Woolf, C. J. (2000). Pain Mechanisms and Management: A Central Perspective. *The Clinical Journal of Pain*, 16, S144-156.
- Mansvelder, H. D., van Aerde, K. I., Couey, J. J., & Brussaard, A. B. (2006). Nicotinic modulation of neuronal networks: from receptors to cognition. *Psychopharmacology*, 184(3-4), 292-305. doi:10.1007/s00213-005-0070-z
- Marks, M. J., Pauly, J. F. L., Heinemann, F., Collins, A. C., Deneris, E. S., Hermans-borgmeyer, I., & Gross, D. (1992). Nicotine Binding and Nicotinic Nicotine Treatment Receptor Subunit RNA after Chronic, (July).
- Marrero, M. B., & Bencherif, M. (2009). Convergence of alpha 7 nicotinic acetylcholine receptor-activated pathways for anti-apoptosis and anti-inflammation: central role for JAK2 activation of STAT3 and NF-kappaB. *Brain research*, 1256, 1-7. Elsevier B.V. doi:10.1016/j.brainres.2008.11.053
- Marrero, M. B., Bencherif, M., Lippiello, P. M., & Lucas, R. (2011). Application of alpha7 nicotinic acetylcholine receptor agonists in inflammatory diseases: an overview. *Pharmaceutical research*, 28(2), 413-6. doi:10.1007/s11095-010-0283-7
- Martin, W. J., Gupta, N. K., Loo, C. M., Rohde, D. S., & Basbaum, a I. (1999a). Differential effects of neurotoxic destruction of descending noradrenergic pathways on acute and persistent nociceptive processing. *Pain*, 80(1-2), 57-65. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/10204718>
- Martin, W. J., Gupta, N. K., Loo, C. M., Rohde, D. S., & Basbaum, a I. (1999b). Differential effects of neurotoxic destruction of descending noradrenergic pathways on acute and persistent nociceptive processing. *Pain*, 80(1-2), 57-65. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/10204718>
- Marubio, L.M., del Mar Arroyo-Jimenez, M., Cordero-Erausquin, M., Léna, C., Le Novère, N., de Kerchove d'Exaerde, A., Huchet, M., Damaj, M.I., & Changeux, J.P. (1999). Reduced antinociception in mice lacking neuronal nicotinic receptor subunits. *Nature*, 398(6730), 805-810.
- Matson, D., McDermott, J.S., Knop, J., Gaida, K., Cao, L., Waldon, D., Albrecht, B.K., Boezio,

- A.A., Copeland, K.W., Harmange, J.C., Springer, S.K., Malmberg, A.B., McDonough, S.I. (2010). Pharmacological effects of nonselective and subtype-selective nicotinic acetylcholine receptor agonists in animal models of persistent pain. *Pain*, 149(1), 33-49.
- Mattila, M.J., Ahtee, L., Saarnivaara, L. (1968). The analgesic and sedative effects of nicotine in white mice, rabbits and golden hamsters. *Annales Medicinae Experimentalis et Biologiae Fenniae*, 46(1), 78-84.
- McGehee, D. S. (1999). Molecular Diversity of Neuronal Nicotinic Acetylcholine Receptors. *Annals of the New York Academy of Sciences*, 868, 565-577.
- McLean, S. L., Idris, N., Grayson, B., Gendle, D., Mackie, C., Lesage, A., Pemberton, D. J., et al. (2011). PNU-120596, a positive allosteric modulator of  $\alpha 7$  nicotinic acetylcholine receptors, reverses a sub-chronic phencyclidine-induced cognitive deficit in the attentional set-shifting task in female rats. *Journal of psychopharmacology (Oxford, England)*. doi:10.1177/0269881111431747
- Measuring, F. O. R., & Pain, S. O. F. (1940). THRESHOLD : OBSERVATIONS ON SPATIAL, (12), 649-657.
- Medhurst, S. J., Hatcher, J. P., Hille, C. J., Bingham, S., Clayton, N. M., Billinton, A., & Chessell, I. P. (2008). Activation of the  $\alpha 7$ -nicotinic acetylcholine receptor reverses complete Freund adjuvant-induced mechanical hyperalgesia in the rat via a central site of action. *The journal of pain : official journal of the American Pain Society*, 9(7), 580-7. doi:10.1016/j.jpain.2008.01.336
- Menegazzi, M., Di Paola, R., Mazzon, E., Genovese, T., Crisafulli, C., Dal Bosco, M., Zou, Z., Suzuki, H., Cuzzocrea, S. (2008). Glycyrrhizin attenuates the development of carrageenan-induced lung injury in mice. *Pharmacological Research*, 58(1), 22-31.
- Millan, M. J. (2002). Descending control of pain. *Progress in neurobiology*, 66(6), 355-474. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/12034378>
- Millar, N. S., & Gotti, C. (2009). Diversity of vertebrate nicotinic acetylcholine receptors. *Neuropharmacology*, 56(1), 237-46. Elsevier Ltd. doi:10.1016/j.neuropharm.2008.07.041
- Min, S.-W., Kim, N.-J., Baek, N.-I., & Kim, D.-H. (2009). Inhibitory effect of eupatilin and jaceosidin isolated from *Artemisia princeps* on carrageenan-induced inflammation in mice. *Journal of ethnopharmacology*, 125(3), 497-500. doi:10.1016/j.jep.2009.06.001
- Moaddel, R., Jozwiak, K., & Wainer, I. W. (2007). Allosteric modifiers of neuronal nicotinic acetylcholine receptors: new methods, new opportunities. *Medicinal research reviews*, 27(5), 723-53. doi:10.1002/med.20091
- Morisseau, C., Inceoglu, B., Schmelzer, K., Tsai, H.-J., Jinks, S. L., Hegedus, C. M., & Hammock, B. D. (2010). Naturally occurring monoepoxides of eicosapentaenoic acid and

docosahexaenoic acid are bioactive antihyperalgesic lipids. *Journal of lipid research*, 51(12), 3481-90. doi:10.1194/jlr.M006007

- Mueller, M., Leonhard, C., Wacker, K., Ringelstein, E.B., Okabe, M., Hickey, W.F., Kiefer, R. (2003) Macrophage response to peripheral nerve injury: the quantitative contribution of resident and hematogenous macrophages. *Laboratory Investigation*, 83(2), 175-185.
- Munro, G., Hansen, R. R., Erichsen, H. K., Timmermann, D. B., Christensen, J. K., & Hansen, H. H. (2012). The  $\alpha 7$  nicotinic ACh receptor agonist compound B and positive allosteric modulator PNU-120596 both alleviate inflammatory hyperalgesia and cytokine release in the rat. *British journal of pharmacology*. doi:10.1111/j.1476-5381.2012.02003.x
- Negus, S. S., Vanderah, T. W., Brandt, M. R., Bilsky, E. J., Becerra, L., & Borsook, D. (2006). Preclinical Assessment of Candidate Analgesic Drugs : Recent Advances and Future Challenges, 319(2), 507-514. doi:10.1124/jpet.106.106377.wide
- Ng, H. J., Whittemore, E. R., Tran, M. B., Hogenkamp, D. J., Broide, R. S., Johnstone, T. B., Zheng, L., et al. (2007). Nootropic  $\alpha 7$  nicotinic receptor allosteric modulator derived from GABA A receptor modulators. *Sciences-New York*, 104(19).
- Ortiz, N. C., O'Neill, H. C., Marks, M. J., & Grady, S. R. (2012). Varenicline Blocks  $\beta 2$ -nAChR-Mediated Response and Activates  $\beta 4$ -nAChR-Mediated Responses in Mice In Vivo. *Nicotine & Tobacco Research Advance Access*, [Epub ahead of time], 1-9.
- Ossipov, M. H., Harris, S., Lloyd, P., & Messineo, E. (1990). An isobolographic analysis of the antinociceptive effect of systemically and intrathecally administered combinations of clonidine and opiates. *The Journal of pharmacology and experimental therapeutics*, 255(3), 1107-16. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/2262895>
- Ostelo, R. W. J. G., & de Vet, H. C. W. (2005). Clinically important outcomes in low back pain. *Best practice & research. Clinical rheumatology*, 19(4), 593-607. doi:10.1016/j.berh.2005.03.003
- Overstreet, D. H., Daws, L. C., Schiller, G. D., Orbach, J., & Janowsky, D. S. (1998). Cholinergic/serotonergic interactions in hypothermia: implications for rat models of depression. *Pharmacology, biochemistry, and behavior*, 59(4), 777-85. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/9586831>
- Papke, R L, Meyer, E., Nutter, T., & Uteshev, V. V. (2000). Alpha7 Receptor-Selective Agonists and Modes of Alpha7 Receptor Activation. *European journal of pharmacology*, 393(1-3), 179-95. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/10771012>
- Papke, Roger L, Kem, W. R., Soti, F., Lo, G. Y., & Horenstein, N. A. (2009). Activation and Desensitization of Nicotinic Alpha 7-type Acetylcholine Receptors by Benzylidene Anabaseines and Nicotine. *Pharmacology*, 329(2), 791-807. doi:10.1124/jpet.108.150151.receptors.

- Papke, Roger L, & Porter Papke, J. K. (2002). Comparative pharmacology of rat and human alpha7 nAChR conducted with net charge analysis. *British journal of pharmacology*, 137(1), 49-61. doi:10.1038/sj.bjp.0704833
- Papke, Roger L, Wecker, L., & Stitzel, J. A. (2010). Activation and Inhibition of Mouse Muscle and Neuronal Nicotinic Acetylcholine Receptors Expressed in *Xenopus* Oocytes, 333(2), 501-518. doi:10.1124/jpet.109.164566.therapeutics.
- Parikh, V., & Sarter, M. (2006). Cortical choline transporter function measured in vivo using choline-sensitive microelectrodes: clearance of endogenous and exogenous choline and effects of removal of cholinergic terminals. *Journal of neurochemistry*, 97(2), 488-503. doi:10.1111/j.1471-4159.2006.03766.x
- Paterson, D., & Nordberg, a. (2000). Neuronal nicotinic receptors in the human brain. *Progress in neurobiology*, 61(1), 75-111. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/10759066>
- Pavlov, V. a, & Tracey, K. J. (2004). Neural regulators of innate immune responses and inflammation. *Cellular and molecular life sciences : CMLS*, 61(18), 2322-31. doi:10.1007/s00018-004-4102-3
- Portenoy, R. K., Ugarte, C., Fuller, I., & Haas, G. (2004). Population-based survey of pain in the United States: differences among white, African American, and Hispanic subjects. *The journal of pain : official journal of the American Pain Society*, 5(6), 317-28. doi:10.1016/j.jpain.2004.05.005
- Rashid, Md Haronur, & Ueda, H. (2002). Neuropathy-specific analgesic action of intrathecal nicotinic agonists and its spinal GABA-mediated mechanism. *Brain research*, 953(1-2), 53-62. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/12384238>
- Rashid, Md Harunor, Furue, H., Yoshimura, M., & Ueda, H. (2006). Tonic inhibitory role of alpha4beta2 subtype of nicotinic acetylcholine receptors on nociceptive transmission in the spinal cord in mice. *Pain*, 125(1-2), 125-35. doi:10.1016/j.pain.2006.05.011
- Receptors, N., Gerzanich, V., Wang, F. A. N., Kuryatov, A., & Lindstrom, J. O. N. (1998).  $\alpha 5$  Subunit Alters Desensitization , Pharmacology ,  $Ca^{2+}$  Permeability and  $Ca^{2+}$  Modulation of Human Neuronal  $\alpha 3$ , 286(1), 311-320.
- Ren, K., Dubner, R. (1993) NMDA receptor antagonists attenuate mechanical hyperalgesia unilateral inflammation of the hindpaw. *Neuroscience Letters*, 163(1), 22-26.
- Ren, K., & Dubner, R. (1996). Enhanced descending modulation of nociception in rats with persistent hindpaw inflammation. *Journal of neurophysiology*, 76(5), 3025-37. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/8930252>

- Roach, J. T., & Sufka, K. J. (2003). Characterization of the chick carrageenan response. *Brain Research*, 994(2), 216-225. doi:10.1016/j.brainres.2003.09.038
- Rogers, S.W., Baker, T., and Gould, T. (2007) Chapter 3: Mouse Models and the Genetics of Nicotine Addiction. In: NCI Monograph 22, Phenotypes and Endophenotypes: Foundations for Genetic Studies of Nicotine Use and Dependence.
- Roncarati, R., Seredenina, T., Jow, B., Jow, F., Papini, S., Kramer, A., Bothmann, H., et al. (2008). Functional properties of alpha7 nicotinic acetylcholine receptors co-expressed with RIC-3 in a stable recombinant CHO-K1 cell line. *Assay and drug development technologies*, 6(2), 181-93. doi:10.1089/adt.2007.120
- Rosas-Ballina, M., & Tracey, K. J. (2009). Cholinergic control of inflammation. *Journal of internal medicine*, 265(6), 663-79. doi:10.1111/j.1365-2796.2009.02098.x
- Rowbotham, M. C., Duan, W. R., Thomas, J., Nothaft, W., & Backonja, M.-M. (2009). A randomized, double-blind, placebo-controlled trial evaluating the efficacy and safety of ABT-594 in patients with diabetic peripheral neuropathic pain. *Pain*, 146(3), 245-52. International Association for the Study of Pain. doi:10.1016/j.pain.2009.06.013
- Rowley, T J, McKinstry, a, Greenidge, E., Smith, W., & Flood, P. (2010). Antinociceptive and anti-inflammatory effects of choline in a mouse model of postoperative pain. *British journal of anaesthesia*, 105(2), 201-7. doi:10.1093/bja/aeq113
- Rowley, Thomas J, & Flood, P. (2008). Isoflurane prevents nicotine-evoked norepinephrine release from the mouse spinal cord at low clinical concentrations. *Anesthesia and analgesia*, 107(3), 885-9. doi:10.1213/01.ane.0000287646.85834.1a
- Rueter, L. E., Meyer, M. D., & Decker, M. W. (2000). Spinal mechanisms underlying A-85380-induced effects on acute thermal pain. *Brain research*, 872(1-2), 93-101. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/10924680>
- Sack, R., Gochberg-Sarver, A., Rozovsky, U., Kedmi, M., Rosner, S., & Orr-Urtreger, A. (2005). Lower core body temperature and attenuated nicotine-induced hypothermic response in mice lacking the beta4 neuronal nicotinic acetylcholine receptor subunit. *Brain research bulletin*, 66(1), 30-6. doi:10.1016/j.brainresbull.2005.02.032
- Sakoori, K., & Murphy, N. P. (2009). Enhanced nicotine sensitivity in nociceptin/orphanin FQ receptor knockout mice. *Neuropharmacology*, 56(5), 896-904. Elsevier Ltd. doi:10.1016/j.neuropharm.2009.01.016
- Sarter, M., & Parikh, V. (2005). Choline transporters, cholinergic transmission and cognition. *Nature reviews. Neuroscience*, 6(1), 48-56. doi:10.1038/nrn1588
- Sattelle, D. B., Buckingham, S. D., Akamatsu, M., Matsuda, K., Pienaar, I. S., Pienaar, I., Jones, A. K., et al. (2009). Comparative pharmacology and computational modelling yield



- insights into allosteric modulation of human alpha7 nicotinic acetylcholine receptors. *Biochemical pharmacology*, 78(7), 836-43. doi:10.1016/j.bcp.2009.06.020
- Schmelz, M., Schmidt, R., Bickel, a, Handwerker, H. O., & Torebjörk, H. E. (1997). Specific C-receptors for itch in human skin. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 17(20), 8003-8. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/9315918>
- Séguéla, P., Wadiche, J., Dineley-Miller, K., Dani, J. a, & Patrick, J. W. (1993). Molecular cloning, functional properties, and distribution of rat brain alpha 7: a nicotinic cation channel highly permeable to calcium. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 13(2), 596-604. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/7678857>
- Serra, J. (1999). Overview of neuropathic pain syndromes. *Acta Neurologica Scandinavica Supplementum*, 173, 7-11.
- Shibata, M., Ohkubo, T., Takahashi, H., Inoki, R. (1989). Modified formalin test: characteristic biphasic pain response. *Pain*, 38: 347-352.
- Stitzel, J.A., Blanchette, J.M., Collins, A.C. (1998). Sensitivity to the seizure inducing effects of nicotine is associated with strain-specific variants of the alpha 5 and alpha 7 nicotinic receptor subunit genes. *Journal of Pharmacology and Experimental Therapeutics*, 284, 1104-1111.
- Tallarida, R.J. (2000). Drug Synergism and Dose-Effect Analysis. Chapman & Hall/CRC Press, Boca Raton, FL.
- Thomsen, M. S., Hansen, H. H., Timmerman, D. B., & Mikkelsen, J. D. (2010). Cognitive improvement by activation of alpha7 nicotinic acetylcholine receptors: from animal models to human pathophysiology. *Current pharmaceutical design*, 16(3), 323-43. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/20109142>
- Thomson, A.W., Fowler, E.F. (1981). Carrageenan: a review of its effects on the immune system. *Agents and Actions*, 11, 265-273.
- Timmermann, D. B., Grønlien, J. H., Kohlhaas, K. L., Nielsen, E. Ø., Dam, E., Jørgensen, T. D., Ahring, P. K., et al. (2009). An Allosteric Modulator of the  $\alpha$ 7 Nicotinic Acetylcholine Receptor Possessing Cognition-Enhancing Properties in Vivo. *Pharmacology*. doi:10.1124/jpet.107.120436.vidual
- Tjølsen, A., Berge, O.G., Hunskar, S., Rosland, J.H., Hole, K. (1992). The formalin test: an evaluation of the method. *Pain*, 51, 5-17.
- Ulloa, L. (2005). The vagus nerve and the nicotinic anti-inflammatory pathway. *Nature reviews. Drug discovery*, 4(8), 673-84. doi:10.1038/nrd1797

- Vincler, M. (2005). Neuronal nicotinic receptors as targets for novel analgesics. *Expert opinion on investigational drugs*, 14(10), 1191-8. doi:10.1517/13543784.14.10.1191
- Vranklen, J.H. (2009) Mechanisms and treatment of neuropathic pain. *Central nervous system agents in medicinal chemistry*, 9, 71-80.
- Wada, E., Wada, K., Boulter, J. I. M., Deneris, E., Heinemann, S., Patrick, J. I. M., & Swanson, L. W. (1989). and Beta2 Neuronal Nicotinic Receptor n the Central Nervous Subunit m R N h i System : A Hybridization Histochemical Study i n the Rat. *Journal of Comparative Neurology*, 336.
- Walker, K., Fox, a J., & Urban, L. a. (1999). Animal models for pain research. *Molecular medicine today*, 5(7), 319-21. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/10498437>
- Walker, S.M., Grafe, M., Yaksh, T.L. (2012). Intrathecal Clonidine in the Neonatal Rat: Dose-Dependent Analgesiaand Evaluation of Spinal Apoptosis and Toxicity. *Anesthesia and Analgesia* [Epub ahead of print].
- Wang, D.-wei, Zhou, R.-bin, & Yao, Y.-ming. (2009). Role of cholinergic anti-inflammatory pathway in regulating host response and its interventional strategy for inflammatory diseases. *Chinese Journal of Traumatology English Edition*, 12(6), 355-364. The Editorial Board of Biomedical and Environmental Sciences. doi:10.3760/cma.j.issn.1008-1275.2009.06.007
- Wang, F., Xu, S., Shen, X., Guo, X., Peng, Y., & Yang, J. (2011). Spinal macrophage migration inhibitory factor is a major contributor to rodent neuropathic pain-like hypersensitivity. *Anesthesiology*, 114(3), 643-59. doi:10.1097/ALN.0b013e31820a4bf3
- Wang, L. X., & Wang, Z. J. (2003). Animal and cellular models of chronic pain. *Advanced drug delivery reviews*, 55(8), 949-65. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/12935939>
- Wang, Y., Su, D.-M., Wang, R.-H., Liu, Y., & Wang, H. (2005). Antinociceptive effects of choline against acute and inflammatory pain. *Neuroscience*, 132(1), 49-56. doi:10.1016/j.neuroscience.2004.12.026
- Winter, C.A., Risley, E.A., Nuss, G.W. (1962). Carrageenan-induced edema in hind paw of the rat as an assay for anti-inflammatory drugs. *Proceedings of the Society for Experimental Biology and Medicine*, 11, 544-547.
- Yalcin, I., Charlet, A., Cordero-Erausquin, M., Tessier, L.-H., Picciotto, M. R., Schlichter, R., Poisbeau, P., et al. (2011). Nociceptive thresholds are controlled through spinal  $\beta(2)$ -subunit-containing nicotinic acetylcholine receptors. *Pain*, 152(9), 2131-7. International Association for the Study of Pain. doi:10.1016/j.pain.2011.05.022



- Young, T., Wittenauer, S., Parker, R., & Vincler, M. (2008). Peripheral nerve injury alters spinal nicotinic acetylcholine receptor pharmacology. *European journal of pharmacology*, 590(1-3), 163-9. doi:10.1016/j.ejphar.2008.06.020
- Zhu, C. Z., Chin, C.-L., Rustay, N. R., Zhong, C., Mikusa, J., Chandran, P., Salyers, A., et al. (2011). Potentiation of analgesic efficacy but not side effects: co-administration of an  $\alpha 4\beta 2$  neuronal nicotinic acetylcholine receptor agonist and its positive allosteric modulator in experimental models of pain in rats. *Biochemical pharmacology*, 82(8), 967-76. doi:10.1016/j.bcp.2011.05.007
- Zhuo, M. (2007). Neuronal mechanism for neuropathic pain. *Molecular pain*, 3, 14. doi:10.1186/1744-8069-3-14
- de Jonge, W. J., & Ulloa, L. (2007). The alpha7 nicotinic acetylcholine receptor as a pharmacological target for inflammation. *British journal of pharmacology*, 151(7), 915-29. doi:10.1038/sj.bjp.0707264
- del Barrio, L., Egea, J., León, R., Romero, A., Ruiz, A., Montero, M., Alvarez, J., et al. (2011). Calcium signalling mediated through  $\alpha 7$  and non- $\alpha 7$  nAChR stimulation is differentially regulated in bovine chromaffin cells to induce catecholamine release. *British journal of pharmacology*, 162(1), 94-110. doi:10.1111/j.1476-5381.2010.01034.x
- van Maanen, M. a, Lebre, M. C., van der Poll, T., LaRosa, G. J., Elbaum, D., Vervoordeldonk, M. J., & Tak, P. P. (2009). Stimulation of nicotinic acetylcholine receptors attenuates collagen-induced arthritis in mice. *Arthritis and rheumatism*, 60(1), 114-22. doi:10.1002/art.24177